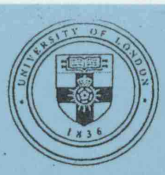

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The Role of Ovarian Follicular Antioxidants and Reactive Oxygen Species in Human Female Reproduction

Oluseye Adelani Oyawoye

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Medicine

University College London

2005

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Dedication

This work is dedicated to my wife Wemi, my children

Delani, Dolapo and Laolu, and my late parents

ABSTRACT

Infertility affects about 15% of couples and in many cases the aetiology is easily identified. Aetiological diagnosis is more difficult in about 15% of females, when infertility is related to less clearly identifiable factors such as luteal phase deficiency and endometriosis. It has been suggested that oxidative stress may interfere with female fertility, analogous to the situation in males, and may play a role in some cases of unexplained fertility. This study was designed to evaluate the role of follicular fluid oxidative stress in female reproduction. Follicular fluid total antioxidant capacity (TAC) and TAC loss, indirect measures of oxidative stress, were related to early reproductive outcome, specifically oocyte recovery, fertilisation, and subsequent embryo viability after 3 days in culture.

Follicular TAC was assayed in a pilot study using two methods - the trolox equivalent antioxidant capacity (TEAC) assay and the ferric reducing/antioxidant power (FRAP) assay - to determine the optimum assay for TAC. Good correlation was observed between the assays. The FRAP assay methodology was robust, easier, cheaper and quicker to perform, and was therefore used to determine follicular fluid TAC in the rest of the study.

Follicular fluid TAC was unrelated to the presence of an oocyte in the source follicle. Oocytes from follicles with lower TAC tended to fail to fertilise, whereas oocytes from follicles that exhibited mid-range and higher TAC were fertilisation competent. Furthermore, high follicular TAC at oocyte retrieval was associated with embryo non-viability, whereas mid-range follicular TAC values were associated with embryo viability after 3 days. Outside of the IVF setting, in women undergoing natural

menstrual cycles (*i.e.* no exposure to hormonal preparations), follicular TAC was similar in sub-fertile women and in fertile controls.

Follicular fluid proteins were estimated by Bradford assay because of the contribution of their sulphydryl group to antioxidant activity. Follicular fluid sulphydryl content was also correlated with TAC. The total follicular protein concentration was relatively consistent (37.5 ± 6.1 mg/ml). Sulphydryl content of follicular fluid corresponded to about 40% of TAC values. No correlation existed between protein and TAC, indicating that variations in TAC values were not just due to changes in protein. Follicular total protein concentration had no influence on reproductive outcome.

Follicular fluid cell density varied widely between patients and between different follicles from the same patient. There was no correlation between follicular fluid cell density and TAC.

These results suggest that the role of oxygen free radicals and antioxidants in female reproduction are complex. A lesser ability to defend against free radicals within the follicle is detrimental to oocyte fertilisation, at least in the IVF setting. It is possible such oocytes suffer free radical damage during their development, which compromises fertilisation competence. Excessive TAC however impairs subsequent embryo viability, suggesting that free radicals are also required for some oocyte maturation processes which influence subsequent developmental competence, which if excessively quenched impair viability. The use of exogenous antioxidants to improve fertility in women cannot yet be justified.

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Finally, I am very grateful to my wife and sons who were very supportive and understanding.

ABBREVIATIONS

AscH, Ascorbate

FRAP, Ferric Reducing/Antioxidant Power (FRAP)

GPx, glutathione peroxidase

GSH, glutathione

GSSH, oxidized glutathione

$L^{\bullet}$, lipid alkyl radicals

$LO^{\bullet}$, lipid alkoxyl radicals

$LOO^{\bullet}$, lipid peroxyl radical

LOOH, lipid hydropeoxides

$O_2^{\bullet -}$, superoxide

ONOO-, Peroxynitrite

PUFA, polyunsaturated fatty acids

ROO•, Peroxyl radical

ROS, reactive oxygen species

SOD, superoxide dismutase

TBARS, thiobarbituric acid reactive substances

TEAC, Trolox Equivalent Antioxidant Capacity

UH3, Uric acid

Chapter 1

Introduction

Outline

The role of oxygen free radicals and reactive oxygen species (ROS) in human female reproduction has not been conclusively established. In this thesis, human follicular fluid total antioxidant capacity (TAC) was investigated as an indirect measure of the activity of oxygen free radicals and ROS in the follicle. TAC parameters were then related to early reproductive outcome after in vitro fertilisation (IVF). The first section of this introductory chapter summarises the physiology of human female reproduction and reviews the problem of infertility. The second section provides an extensive review of the chemistry of oxygen free radicals and ROS in body tissues, as well as their roles in physiological and pathological processes.

1.1 The Physiology of Human Female Reproduction

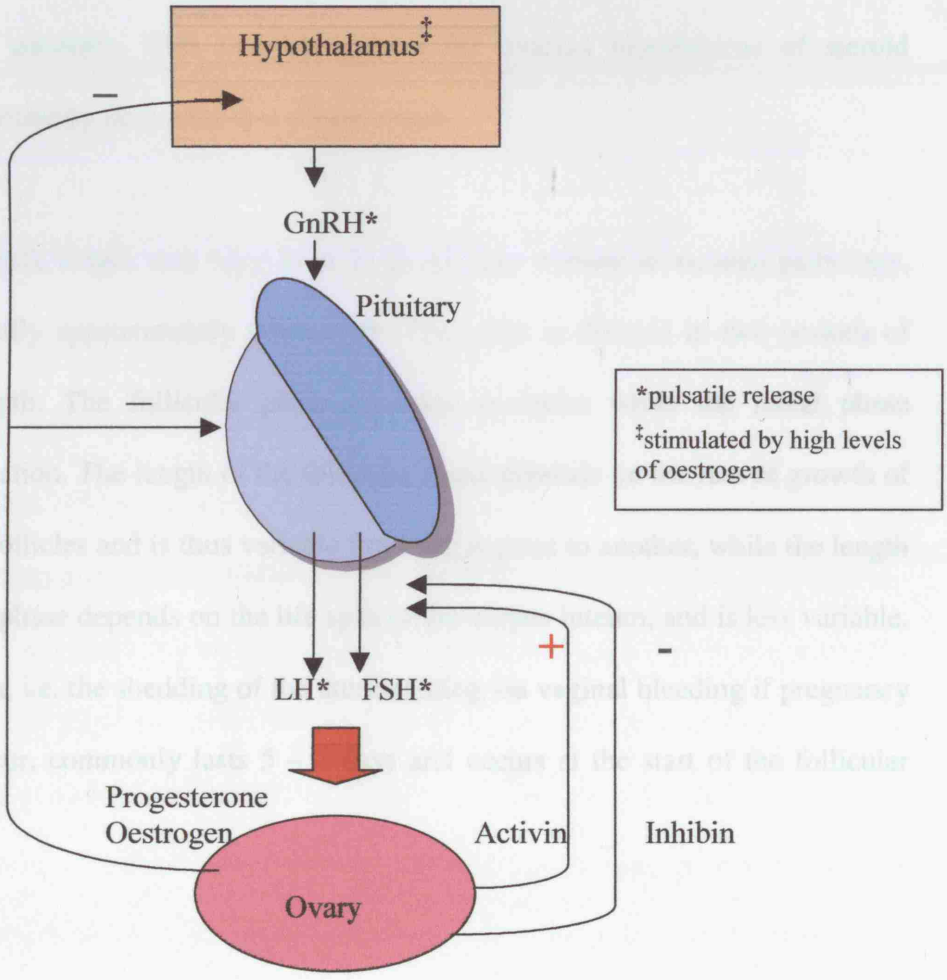
1.1.1 *The Ovary*

The ovaries are ovoid structures, measuring approximately 3 x 1.5 x 1 cm, composed of dense fibrous tissue in which primary and developing follicles are embedded. The ovary usually lies with its long axis oblique and its tubal extremity uppermost and medial, attached to the upper angle of the uterus by the ovarian ligament. Macroscopically, the ovary consists of a capsule, a cortex and a medullary region. The capsule consists of an outer germinal epithelium (a single layer of cuboidal or squamous cells) and the fibrous tunica albuginea lies below. The central medullary region contains connective tissue and the main blood vessels. The peripheral portion surrounding the medulla, the cortex, contains ovarian follicles embedded in connective tissue.

The ovaries serve the functions of sex hormone production and gametogenesis in female mammalian reproduction. Ovarian follicles contain an oocyte, surrounded by

a layer of granulosa epithelial cells on a basement membrane, coated with an outer layer of theca cells. The theca cells express luteinising hormone (LH) receptors, and produce androgens in response to LH. The androgens cross the basement membrane to reach the granulosa cells, which express aromatase activity and therefore convert androgens to oestrogens, predominantly oestradiol, which is the primary female sex hormone. Aromatase is a follicle stimulating hormone (FSH) induced enzyme and granulosa cells express FSH receptors. Gonadotrophin release is also influenced by the central nervous system and by hormones such as oestrogen, progesterone, inhibin and activin (Figure 1.1).

Figure 1.1: The Hypothalamic-Pituitary-Ovarian axis showing interactions with activin and inhibin



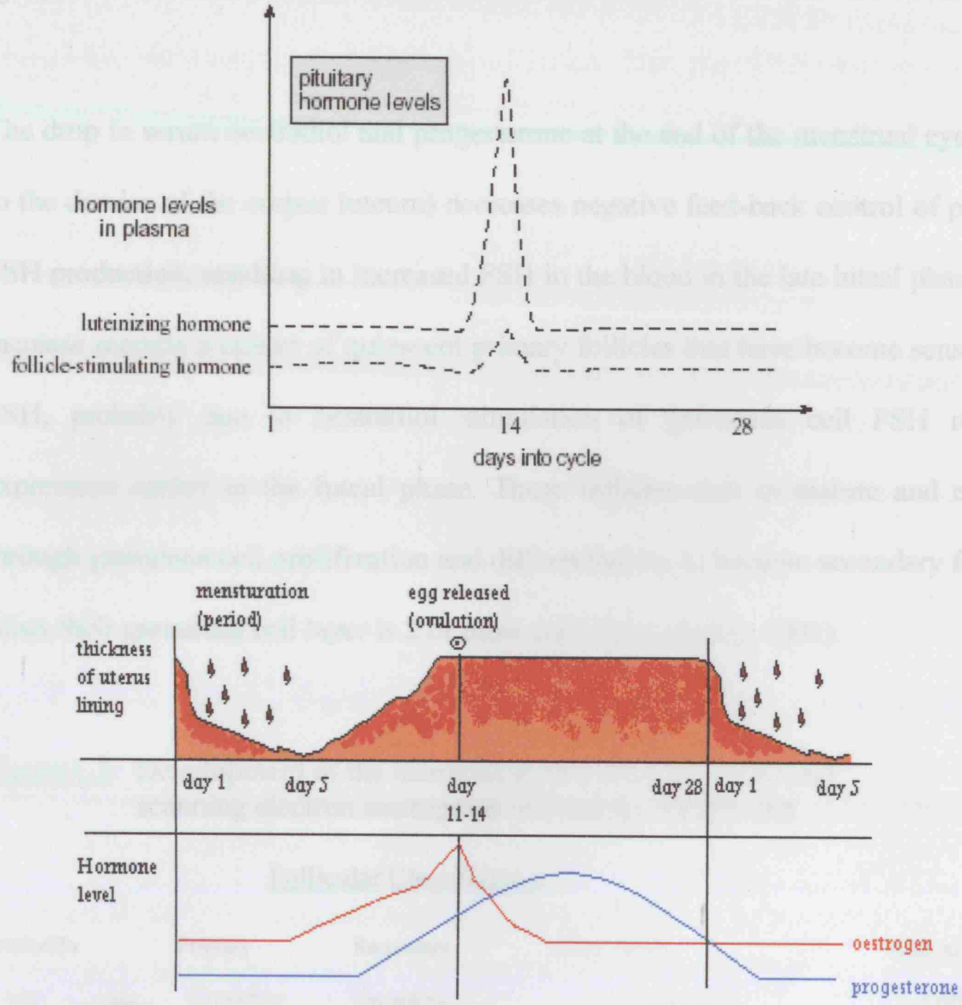
The gonadotrophins, FSH and LH act synergistically to promote follicular growth and development, and the regulated synthesis of gonadal steroid hormones seen in the human menstrual cycle. Both FSH and LH are glycoproteins secreted in a pulsatile fashion from the anterior pituitary, in response to hypothalamus-derived gonadotrophin releasing hormone (GnRH).

1.1.2 *The menstrual cycle and ovulation*

The menstrual cycle (Figure 1.2) results in periodic ovulation to produce the oocyte, the female gamete, and preparation of the female body to allow oocyte fertilisation and zygote implantation, and to support the resulting pregnancy. The ovaries, uterus and cervix undergo substantial changes during the cycle. The menstrual cycle is regulated at the endocrine level by the cyclical release of FSH, LH and prolactin from the anterior pituitary. FSH and LH govern the ovarian biosynthesis of steroid hormones, primarily oestradiol and progesterone.

Menstrual cycle length may vary from 21 to 35 days without associated pathology, but is normally approximately one month. The cycle is divided in two periods of unequal length. The follicular phase precedes ovulation while the luteal phase follows ovulation. The length of the follicular phase depends on the rate of growth of the ovarian follicles and is thus variable from one woman to another, while the length of the luteal phase depends on the life span of the corpus luteum, and is less variable. Menstruation, i.e. the shedding of the uterine lining via vaginal bleeding if pregnancy does not occur, commonly lasts 5 – 7 days and occurs at the start of the follicular phase.

Figure 1.2: The Menstrual Cycle



http://www.chadevans.co.uk/gcsbiology/images/hi-resm2/mcycle(normal).tif

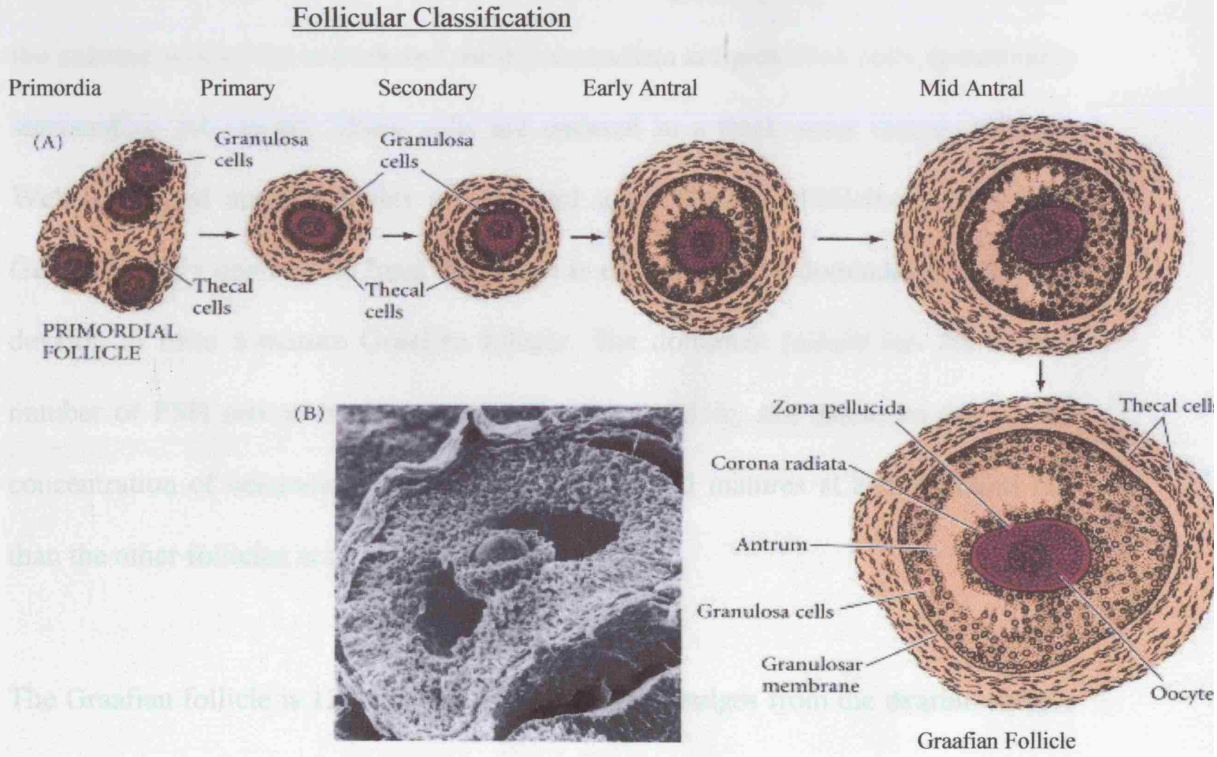
1.1.3 *Follicular development*

Ovarian primordial follicles form in the 7th month of female embryonic development and consist of a primary oocyte surrounded by a layer of flattened follicular epithelial cells. By birth these primordial follicles have developed into primary follicles – the primary oocyte enlarges while the follicular cells proliferate and differentiate into stratified granulosa epithelial cells within a basement membrane. A thecal cell layer forms the outside of the primary follicle (Figure 1.3). Follicular development then

remains static until puberty, after which 5 - 20 follicles mature with each menstrual cycle.

The drop in serum oestradiol and progesterone at the end of the menstrual cycle (due to the demise of the corpus luteum) decreases negative feed-back control of pituitary FSH production, resulting in increased FSH in the blood in the late luteal phase. This increase recruits a cohort of quiescent primary follicles that have become sensitive to FSH, probably due to oestradiol stimulation of granulosa cell FSH receptor expression earlier in the luteal phase. These follicles start to mature and enlarge, through granulosa cell proliferation and differentiation, to become secondary follicles when their granulosa cell layer is 2 or more cells thick (Eppig, 2001).

Figure 1.3: Development of the mammalian ovarian follicle (A) and scanning electron micrograph of a mature follicle (B)



(A) after Carlson, 1981; (B) courtesy of P. Bagavandoss

Over several subsequent menstrual cycles, oestradiol stimulates granulosa FSH receptor expression, while FSH promotes oestrogen receptor expression and sensitivity, both leading to further proliferation. This dual FSH-oestrogen positive feedback interaction within the granulosa cells results in rapid follicular growth, development and maturation. LH and oestradiol also stimulate thecal cell proliferation. The optimal development of pre-antral follicles requires FSH and LH, but intraovarian factors, such as inhibin, activin, interleukin-1 and tumour necrosis factor α and β are also required, acting *via* autocrine and paracrine pathways to modulate follicular development, as discussed in Section 1.1.4. (Fortune and Eppig, 1979; Cortvrindt et al, 1997; Hillier, 2001).

As the follicle grows, it acquires a fluid-filled cavity (the antrum) containing an eccentrically located oocyte and is then referred to as an antral follicle. At this stage in follicular development, the zona granulosa is a layer of granulosa cells that forms the exterior wall of the antrum and the corona radiata are granulosa cells immediately surrounding the oocyte. These cells are encased in a thick outer thecal cell coat. Well-developed antral follicles are referred to as Graafian follicles (Figure 1.3). Generally, only one follicle from the cohort in each cycle (the dominant follicle) will develop to form a mature Graafian follicle. The dominant follicle has the highest number of FSH receptors, the greatest aromatase activity and produces the highest concentration of oestradiol. It therefore develops and matures at a more rapid rate than the other follicles in the cohort.

The Graafian follicle is 12 – 20 mm in diameter and bulges from the ovarian surface from around day 8 of the cycle. The maturing oocyte is within the antrum, covered by the corona radiata and surrounded by follicular fluid. The antrum is lined by a deep

granulosa cell layer, delimited by the basement membrane, and surrounded by the internal and external theca cell layers. By this developmental stage, the oocyte within the Graafian follicle, which had been stalled at the prophase (diplotene) stage of its first meiotic division since foetal life, will have reinitiated and progressed through the first meiotic division, giving rise to a secondary oocyte and the first polar body.

In the cycle in which the dominant follicle reaches maturity, FSH and LH act to stimulate granulosa and theca cell proliferation and follicle maturation throughout the follicular phase. Oestradiol synthesis increases during this time and serum oestradiol levels reach a peak about 24 hr before ovulation (Hackeloer et al, 1979). Oestradiol exerts a complex pattern of feedback control on pituitary LH and FSH secretion. The normal response to oestradiol is a negative feedback effect, but when serum oestradiol is greater than 735 pmol/L for over 60 hours, a positive feedback on LH occurs (Shoupe and Lobo, 1997). LH secretion therefore increases in response to rising plasma oestradiol levels, stimulating greater progesterone production by the dominant follicle from between 12 and 24 hours before ovulation. These changes lead to a rapid and marked surge in LH secretion and an accompanying midcycle FSH peak (March et al, 1979).

The preovulatory LH surge primes the Graafian follicle for ovulation. The Graafian follicle becomes fully mature measuring about 20 mm in diameter and containing a fully-grown oocyte, about 1 million granulosa cells, a theca interna and a band of smooth muscle cells. The oocyte it contains resumes its second meiotic division and ovulation occurs when the oocyte has progressed to prophase II. Oocyte meiosis then arrests at metaphase II and further maturation occurs only if the oocyte is fertilised, otherwise it dies within 24 hours. The LH surge also stimulates the production of

enzymes which degrade extracellular matrix so as to promote ovulatory rupture of the follicle. Furthermore, the LH surge initiates the differentiation of the follicle from one supporting oocyte development and oestrogen synthesis, to one which synthesizes progesterone to maintain the female body in a state permissive for conception, implantation and pregnancy, *i.e.* the corpus luteum. Rupture of the follicle and extrusion of the oocyte surrounded by the cumulus of granulosa cells (ovulation) occur roughly 16 hours after the crest of the LH peak or 32 to 35 hours after the start of the midcycle LH surge.

After ovulation, the empty Graafian follicle completes differentiation into the progesterone secreting corpus luteum, while the rest of the follicular cohort becomes atretic as a result of apoptosis (programmed cell death). The mechanisms triggering the initiation of follicular growth are not completely understood and are currently subject to intense research efforts. The involvement of growth factors and their receptors as well as protooncogenes in the transition of resting follicles to growing follicles is also receiving research consideration.

1.1.4 *Control of follicular development*

It is thought that a variety of growth factors and cytokines have different roles to play in follicular development. Activin and inhibin produced by granulosa cells have important modulator actions on thecal androgen synthesis. Inhibin is an FSH-inducible protein that is likely to play an endocrine role by inhibiting pituitary FSH and LH release. Granulosa cell-derived activin has also been shown to enhance the FSH-supported induction of granulosa cell LH receptors. Also, while activin is inhibitory, inhibin stimulates androgen synthesis (Hillier, 2001). Physiologically, the combination of inhibin B and oestradiol signals to the hypothalamus/pituitary to

suppress FSH secretion, giving an advantage to the dominant follicle over the rest of the cohort follicles. Inhibin B secretion by preantral and small antral follicles is stimulated by FSH, and further enhanced by FSH and IGF-1, during the follicular phase (Fraser et al, 1999; Welt and Schneyer, 2001)

Insulin-like growth factor 1 (IGF-1) receptors present on granulosa and theca cells have a modulating role in human and primate ovarian follicular growth (Lucy, 2000). The IGFs stimulate ovarian function by acting synergistically with gonadotrophins to promote growth and steroidogenesis of ovarian cells (Lucy, 2000). Intrafollicular IGF-I and IGF-II augment LH-stimulated human theca cell androgen production *in vitro,* and these effects are enhanced by inhibin. Androgen produced by theca cells is required as an oestrogen precursor by granulosa cells (Hillier, 1997).

Interleukin-1 is a cytokine produced by activated macrophages and at physiological concentrations appears to have anti-gonadotrophic activity (Van Voorhis, 1999). Other substances that may modulate follicular development include epidermal growth factor, transforming growth factor, nitric oxide and follistatin. These substances therefore affect oocyte growth, fertilisation potential and embryo quality.

There have been many studies investigating the physiological and pathological roles of oxygen free radicals and ROS in the human, but only a few were related to the ovary, oocyte or female reproduction. There are suggestions that these reactive molecules generally have an adverse influence on female fertility, analogous to that reported in the male, but the situation is unclear and requires further investigation (Section 2 of this chapter). The studies in this thesis are focussed on the role of

oxygen radicals in female reproduction with particular reference to antioxidant activity in follicular fluid.

1.1.5 *Fertilisation*

The oocyte released at ovulation is picked up by the fimbria and fertilisation occurs in the ampullary portion of the fallopian tube. Sperm penetration into the oocyte triggers the resumption of meiosis II. The male and female pronuclei fuse in the process called 'syngamy' and pairing of sister chromatids occurs, producing the zygote. The zygote undergoes a series of cleavage divisions to form a blastocyst, consisting of an outer epithelium, the trophectoderm, which later gives rise to placental tissues, and the inner cell mass, from which the foetus forms. The blastocyst stage is observed from about 120 hours after fertilisation (Edwards, 1980). The blastocyst arrives in the uterus 4 - 5 days after ovulation, implantation occurring 2 - 3 days later.

1.2 Infertility

The term infertility is used to describe the situation where a couple of reproductive age does not achieve a spontaneous pregnancy in spite of 'exposure to the risk of pregnancy' over a given period of 12 months (WHO, 2000) or 24 months (NICE Guideline-Fertility, 2004). Simply put, 'the incapability of producing offsprings' (Oxford English Dictionary, 2002). Using the WHO definition, it is estimated that infertility affects between 8 and 15 % of the reproductive population or one in seven couples in the UK (Templeton et al, 1990; Sciarra, 1994; Reproductive Health Outlook, 2002).

In the United Kingdom, Hull et al (1985) studied 708 couples of whom 515 were attending a major gynaecological infertility clinic during 1982 and 1983. A further 172 couples were attending a gynaecological endocrinological clinic, and 21 were referred directly to specialists in male infertility. After excluding couples referred from outside the Bristol area, the average number of couples resident in the Bristol and Weston health district attending specialist infertility clinics in 1982 and 1983 was found to be 472 each year, the calculated incidence of infertility in the district was 17%. In a multicentre survey conducted over 1 year (July 1988 - June 1989) in three regions of France (Thonneau et al, 1991), 1686 couples were investigated for infertility indicating a prevalence of 14.1%. In the US, surveys conducted by the National Centre for Health Statistics in 1988 estimated that 8.4% of women aged 15 - 44 years had infertility problems (Jones and Toner, 1993).

To put the problem of infertility in perspective regarding its incidence relative to other diseases, a cross-sectional survey of the epidemiology of hypertension in England, which investigated a representative sample of 11529 English adults living in non-institutional households, found 37% of adults to be hypertensive according to the new diagnostic criteria (systolic $\geq$ 140 mm Hg or diastolic $\geq$ 90 mm Hg) (Primatesta et al, 2001). The prevalence of diabetes in the UK has approximately doubled in the past 20 years to 2.46% (Harvey et al, 2002), though the prevalence of diabetes among some black and minority ethnic groups in the UK may be as much as 3 - 5 times higher (especially type 2 diabetes) than in Caucasians (Diabetes UK, 2001). Likewise, Ayus-Mateos et al (2001) reported the overall prevalence of depressive disorders in Europe as 8.6%, with the highest rates in urban Ireland and UK. These disorders generally have a lower prevalence rates than that of infertility, which is by no means a less significant health issue considering its associated social,

emotional and health implications, yet they attract considerably more research and clinical investment.

Couples with infertility problems therefore comprise one of the largest groups suffering health problems in our society, deserving of increased medical and research investigation. Though modern technology can provide offspring in about 90 - 95% of cases through couple and donor gamete schemes, relatively few couples can afford these treatments. Thus infertility has become an important socio-economic and political issue (Fishel and Dowell, 2000). Despite the increased opportunities to have children made possible by new technology in assisted conception, the results of a world-wide study of fecundity indicate that in industrialised countries, 3 - 8% of couples have not had a child at the end of their reproductive life, although they desired one (Spira, 1986; Chandra and Stephen, 1998).

1.2.1 *Psychological Aspect of Infertility*

Infertility places intense emotional stress on the male and female partners in a relationship because, for many couples, the ability to bear children is paramount within their relationship. The inability to conceive or give birth threatens gender identity, places one's values and motivations for parenthood, even life, in question, and forces a couple to re-evaluate the meaning of their relationship (Mahlstedt, 1985). Often, an infertility problem may be the first major life crisis met by many couples, who may find that they are unable to cope with its impact. Self-esteem and self-confidence are eroded and the relationship can suffer from blame, guilt, frustration and disappointment (Wright et al, 1991).

1.2.2 *Aetiology of Infertility*

Infertility may result from an abnormality in the female (10 - 30%), the male (25%), or more commonly in both partners. These percentages differ widely depending on the population and facilities available for investigating the aetiology of infertility in each partner.

Male problems are now recognised as a major factor in the aetiology of infertility. These may be in the form of low sperm count, poor motility, abnormal sperm morphology, biochemical dysfunction, antisperm antibodies, obstructive azoospermia and spermatogenic failure. Female factors include ovulation dysfunction and failure, endocrine dysfunction, tubal disease/absence, endometriosis, anatomical abnormalities, poor oocyte quality, chromosome dysfunction and recurrent implantation failure. According to Hull (1996), the main causes of infertility in the Bristol area are ovulatory failure and oligo-amenorrhea (20%), tubal or pelvic infective diseases (20%), endometriosis (10%) and male factor (25%), leaving 25% of cases as 'unexplained'. Spira (1986) compiled the results of seven epidemiological studies carried out in industrialised countries between 1962 and 1983, involving a total of 3956 couples. They found that infertility was due to abnormalities in the women in 60% of couples, the man in 25% of cases and in both partners in the remainder.

1.2.3 *Unexplained Infertility*

Infertility is said to be idiopathic or unexplained when a couple does not conceive and no definite cause of infertility can be diagnosed after a complete evaluation. Despite improved diagnostic techniques, the average incidence of unexplained infertility still ranges from 5% to 28% (Spira, 1986; Wright et al, 1991; Jones and

Toner, 1993; Templeton, 1995; Hull, 1996; Adamson and Baker, 2003). This wide variation may be attributed to selection bias in studies from referral-based infertility units or may reflect other differences between study populations. The incidence of infertility will also be affected by the duration of "risk of exposure to pregnancy" required to make a diagnosis, since using only one year rather than 2 years will result in the inclusion of some subfertile couples, who in fact conceive in the second year. Another important factor is that there is no consensus on which diagnostic tests are essential before reaching a diagnosis of unexplained infertility.

1.2.4 *Other causes of Infertility*

Although some of the causes of female infertility, such as anovulation and tubal blockage are well characterised, others such as cervical factors, luteal-phase deficiency, and immunological factors, require further research (Baird and Wilcox, 1985).

Cigarette smoking has been reported as a possible, though subtler, cause of infertility (Baird and Wilcox, 1985; Phipps et al, 1987; Paszkowski et al, 2002). Phipps et al (1987) compared smoking histories in 901 infertile women and 1264 women admitted for delivery, and found a significant association between cigarette smoking and primary infertility in women. Similarly, in studies by Hull et al (2000), delayed conception was associated with both active and passive smoking in women, even after statistical adjustment for confounding factors. The mechanism by which smoking impacts on fertility in the female is however unclear.

Endometriosis is seen more frequently among women being investigated for infertility (21%) than among those undergoing sterilisation (6%) (RCOG guidelines,

2000). The mechanism of endometriosis-associated infertility is still not fully understood. Moderate or severe endometriosis may cause infertility due to mechanical disruption of ovulation and gamete transport, but the influence of minimal or mild endometriosis on fertility remains controversial. Though there is no difference in the number and quality of oocytes retrieved during IVF, or on fertilisation rates, between women with tubal infertility, endometriosis and unexplained infertility (Lessey, 2000), several studies indicate that endometriosis independently contributes to infertility, decreasing the implantation rate, possibly secondary to endometrial dysfunction or embryotoxic effect of endometriosis related secreted products (Arici et al, 1996; Lessey, 2000). This would suggest that the effects of endometriosis on fertility are not mediated at the level of the oocyte or fertilisation but occur further along in pregnancy. A meta-analysis of published non-randomised control trials suggested that surgical treatment of endometriosis associated with infertility, resulted in higher pregnancy rates than medical treatment or expectant management alone (RCOG guidelines 2000), confirming the results of a previous randomised controlled trial by Marcoux et al (1997).

Genital infections with *Chlamydia trachomatis, Ureaplasma urealyticum* and *Mycoplasma hominis* are more common in infertile couples. Chlamydial infections are associated with at least 50% of cases of acute pelvic inflammatory disease in developed countries and are asymptomatic in 50 - 80% of affected women (Thomas et al, 2000). *C. trachomatis* is an immunogen that stimulates both humoral and cell-mediated immunity, the latter resulting in tissue injury leading to fibrosis, scarring and tubal damage (Land and Evers, 2002). Seroepidemiological studies have confirmed a strong link between chlamydial infections and tubal pathology (Land and Evers, 2002). Genital *C. trachomatis* is found in 2.4% of asymptomatic subfertile

couples (Eggert-Kruse et al, 2003). Heavy *Uroplasma urealyticum* infection causes sperm deformity and reduced motility in men (Nunez-Calonge et al, 1998) but the role of this organism in women is less clear (Witkin et al, 1995).

This study hypothesised that in women, some of the effects of confounding factors on the aetiology of sub-fertility may be mediated through alterations in the activity of oxygen free radicals and reactive oxygen species (ROS) in the preovulatory oocyte environment, as described in the following section.

1.3 Oxygen Free Radicals and Reactive Oxygen Species

Quantum chemistry theory suggests that two electrons are required to form a covalent bond within a molecular orbital. Paired electrons are more stable and the vast majority of biological molecules contain such covalent bonds. When electron pairs split however, the previously covalently bonded molecules or atoms each contain an unpaired electron, and are described as free radicals. These unpaired electrons are highly reactive (thermodynamically unstable) and 'seek out' other electrons with which to pair. Since most electrons exist in a paired state within covalent bonds, free radicals often react with paired electrons and, in so doing, cause covalent bond fission, one of the electrons pairing with the (former) free radical to produce a new covalent bond, while the "odd electron" becomes another free radical. Only when a free radical pairs with another free radical is free radical activity terminated. Apart from the formation of free radicals by the addition of an electron to a neutral atom, free radicals may also be generated by homolysis of covalent bonds, and by the loss of an electron from a neutral atom. ROS, such as hydrogen peroxide (H_2O_2) are not free radicals themselves but are capable of generating free radicals in tissue reactions (Equation 1.1).

Reactive oxygen species are formed by several different mechanisms:

- the interaction of ionising radiation with biological molecules
- as an unavoidable by-product of cellular respiration. Some electrons passing "down" the respiratory chain leak away from the main path (especially as they pass through ubiquinone), directly reducing oxygen molecules to the superoxide anion
- synthesized by enzymes in phagocytic cells including neutrophils and macrophages
    - NADPH oxidase (in both type of phagocytes)
    - myeloperoxidase (in neutrophils only)

Oxygen is required to support life but when inappropriately metabolised, potentially toxic oxygen free radicals are produced, capable of modifying cell function and endangering cell survival. Oxygen free radicals are usually generated within cells as by-products of normal cellular metabolic processes such as in the mitochondrial respiratory chain, phagocytosis and redox based enzyme systems. Eukaryotic cells continuously produce ROS and free radicals, including H_2O_2 and the superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals, as side-products of electron transfer. The hydroxyl free radical is particularly dangerous since it can oxidise and fragment organic molecules. It arises as a product of the reaction between $O_2^{\bullet -}$ and H_2O_2 in a reaction (Fenton's reaction) catalysed by Fe^{2+} (Equation 1.1).

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe^{2+}/Fe^{3+}} OH^{\bullet} + OH^{-} + O_2 \qquad \text{[Equation 1.1]}$$

Fenton (1894) reported this free radical reaction after noting that the addition of ferrous sulphate solution, followed by hydrogen peroxide, to a solution of tartaric acid, produced a violet colour on addition of caustic alkali. Gerschman (1954) reported the formation of oxygen free radicals in mice exposed to hyperbaric oxygen, and associated the formation of excess amount of oxygen free radicals with decreased life span. Because female mice survived longer, the author suggested that oestrogens might have a protective effect which made them less sensitive to the detrimental effects of oxygen free radicals.

In the early 1950s, the toxic effects of treatment with increased oxygen tension led to an estimated 10,000 cases of blindness in newborns due to retrolental fibroplasia (Southom and Powis, 1988). The role of ROS and oxygen free radicals in this process was suggested after the products of lipid auto-oxidation were found (Southom and Powis, 1988). The interactions between free radicals, lipid hydroperoxides and antioxidants have been extensively reviewed (Halliwell and Gutteridge, 1999).

1.3.1 *Formation and Biochemistry of ROS and Oxygen Free Radicals*

Cellular respiration is the process whereby the energy stored in glucose is transferred to adenosine triphosphate (ATP), the main source of metabolic energy at the cellular level. The complete breakdown of glucose to carbon dioxide and water requires the combined metabolic processes of glycolysis, the tricarboxylic acid (TCA) cycle and mitochondrial oxidative phosphorylation (aerobic respiration). Glucose is oxidized and oxygen is reduced to form water, the carbon atoms being released as carbon dioxide (CO_2) (Equation 1.2). Glycolysis produces two moles of ATP per mole of glucose; another 34 moles of ATP are produced by aerobic the TCA cycle and oxidative phosphorylation if oxygen is present.

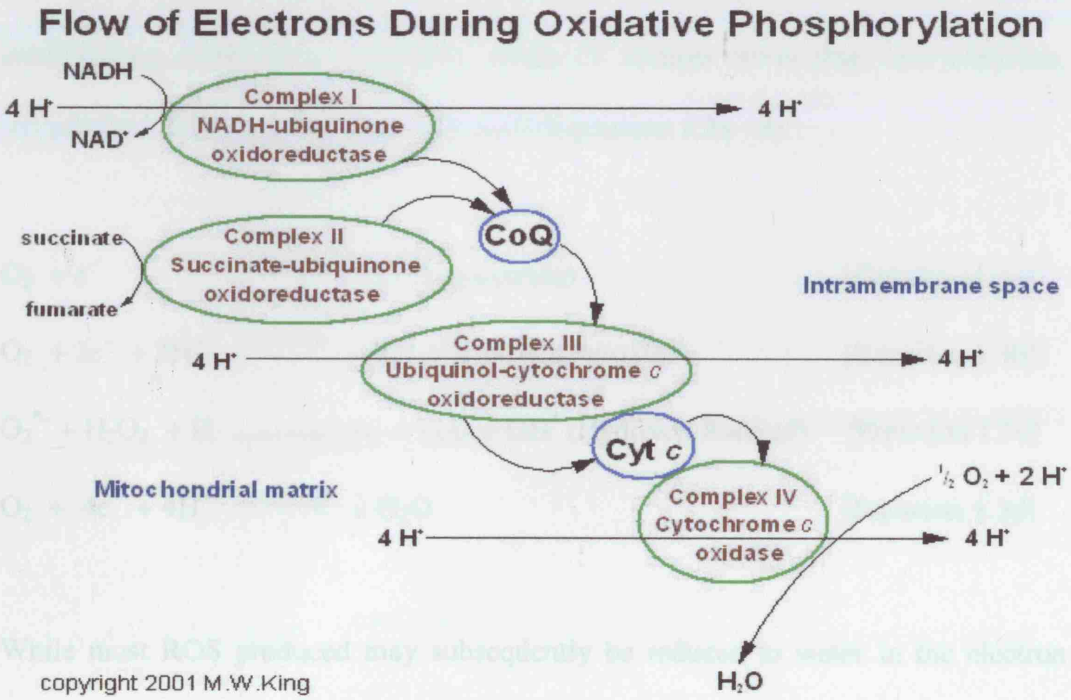
$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \qquad \text{[Equation 1.2]}$$

$$36ADP + 36\ P(i) \qquad 36\ ATP$$

Metabolic energy production requires that food materials be converted to glucose intermediates on the glycolytic pathway or TCA cycle, which are oxidized to CO_2, releasing electrons that are accepted by electron carriers such as nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^+). Reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) are re-oxidized by O_2 in mitochondria in the process of oxidative phosphorylation, producing large amounts of ATP.

Transfer of electrons to oxygen is catalysed in a stepwise fashion *via* the mitochodrial electron transport chain within the inner mitochondrial membrane (Figure 1.4).

Figure 1.4:



(In: http://www.dentistry.leeds.ac.uk/biochem/thcme/oxidativephosphorylation.pdf)

Electrons enter the electron transport chain primarily from NADH to complex I, but can be supplied by $FADH_2$ (produced by succinate dehydrogenase and the glycerol phosphate shuttle), to complex II. The part of the chain that reduces oxygen to water is the terminal complex IV, cytochrome *c* oxidase, which removes an electron from each of 4 reduced (Fe^{2+}- haem) cytochrome *c* molecules, oxidising them to ferric cytochrome *c*. Electrons flow through ubiquinone and the cytochrome proteins of complexes I – IV, such that the energy of their redox potential is slowly released as they pass down the redox gradient to oxygen, but the release is coupled to the transfer of protons out through the inner mitochondrial membrane (Figure 1.4). The electrochemical gradient produced across the inner mitochondrial membrane is used to power ATP synthesis from ADP + phosphate by ATP synthase.

Normally, mitochondria reduce O_2 completely to H_2O in a four-electron reaction catalysed by cytochrome *c* oxidase. When O_2 accepts fewer than four electrons, oxygen free radicals or ROS are generated (Equations 1.3a - d).

$O_2 + e^- \longrightarrow O_2^{\bullet -}$ (Superoxide) [Equation 1.3a]

$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$ (Hydrogen Peroxide) [Equation 1.3b]

$O_2^{\bullet -} + H_2O_2 + H \longrightarrow O_2 + H_2O + OH^{\bullet}$ (Hydroxyl Radical) [Equation 1.3c]

$O_2 + 4e^- + 4H^+ \longrightarrow 2\,H_2O$ [Equation 1.3d]

While most ROS produced may subsequently be reduced to water in the electron transport chain, some "escape" and can react, detrimentally, with various cell components. Leakage of $O_2^{\bullet -}$ during the mitochondrial transfer of electrons to oxygen

(Equation 1.3a; single electron reduction of O_2) represents a major source of ROS and oxygen free radicals within cells. This occurs in at least two locations within the respiratory chain; Complex I NADH/Q oxidoreductase and Complex III ubiquinone-cytochrome *c* oxidoreductase (Figure 1.4).

Superoxide is also formed by enzymatic reactions catalysed by flavin oxidases, xanthine oxidase and monoamine oxidase, as well as by the non-catalytic oxidation of oxyhaemoglobin (containing Fe^{2+}), of which about 3% is converted to methaemoglobin containing Fe^{3+} (Knight, 1998). H_2O_2 is produced by normal non-mitochondrial metabolism especially in peroxisome. The formation of hydroxyl radicals usually occurs via non-enzymatic reduction of H_2O_2 (e.g. by superoxide).

Inflammatory stimuli and chemotactic agonists are capable of stimulating the production of superoxide and related radicals by a tightly regulated, membrane-bound NADPH oxidase in phagocytes. This production of free radicals is associated with an abrupt but transient rise in oxygen consumption, the 'respiratory burst'. The powerful free radical oxidising agents are injected into the phagocytic vacuole, and they allow the phagocytes to kill internalised bacteria. The respiratory burst is characterised by increased glycogenolysis and glucose oxidation, accompanied by a rapid activation of leukocyte NADPH oxidase, which oxidises NADPH and generates superoxide (Equation 1.4):

$$2O_2 + NADPH \xrightarrow{\text{NADPH Oxidase}} 2O_2^{\bullet -} + NADP^+ + H^+ \quad \text{[Equation 1.4]}$$

$O_2^{\bullet-}$ is converted by spontaneous dismutation into H_2O_2, which reacts with Cl^- to form $HOCl^{\bullet}$, catalysed in the neutrophil lysosome by myeloperoxidase. $HOCl^{\bullet}$ is a powerful antimicrobial agent that kills bacteria by halogenation, and lipid peroxidation.

$$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} HOCl^{\bullet} + OH^- \qquad \text{[Equation 1.5]}$$

Activated macrophages and endothelial cells also produce nitric oxide. Though a weak free radical, nitric oxide reacts rapidly with $O_2^{\bullet-}$ to form the extremely reactive peroxynitrite species ($ONOO^-$).

Several biological molecules, such as $FADH_2$, ascorbic acid, adrenalin, and the sulphydryl (thiols) compounds can donate an electron to O_2 to produce $O_2^{\bullet-}$. Most of these auto-oxidation reactions are catalysed by transition metal ions, *e.g.* iron and copper. It is difficult to produce solutions free from contaminating metal ions, thus it is possible that 'auto-oxidation' reactions are metal ion-catalysed *in vitro* (Halliwell and Gutteridge, 1999). Since iron ions and oxygen are freely available *in vivo*, such reactions may occur to produce $O_2^{\bullet-}$ in physiological and pathological situations. It is also probable that body fluids continue to produce free radicals *in vitro*.

1.3.2 *Biological Oxygen Free Radical Reactions*

Though the generation of highly reactive oxygen free radicals is a feature of normal cellular metabolism, their production may be amplified in pathological circumstances, either by cellular or acellular mechanisms. This is exemplified by the reaction between H_2O_2 and Cl^- discussed above in the neutrophil lysosome.

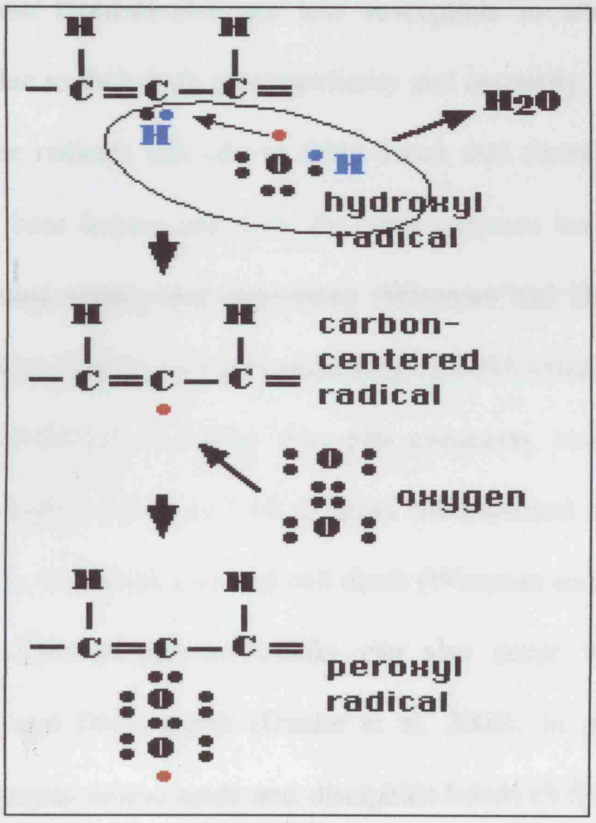
The hydroxyl free radical ($OH^{\bullet}$) is a strong oxidant, which can initiate free radical chain reactions in unsaturated organic molecules, notably polyunsaturated fatty acids (PUFA) in cell membrane lipids, in the process of lipid peroxidation (Figure 1.5). PUFA have carbon-carbon double bonds that are susceptible to $OH^{\bullet}$-mediated abstraction of H atoms, thus initiating lipid peroxidation reactions. Cell membranes are attacked by oxygen free radicals generating fatty acid peroxides, malonaldehyde and other degradation products (Jones et al, 1979). Lipid peroxidation is stimulated by transition metal ions, such as Fe^{2+}/Fe^{3+}, since they boost the generation of $OH^{\bullet}$ from $O_2^{\bullet-}$ and H_2O_2 (Hall, 1997).

Propagation reactions continue the chain reaction - the lipid peroxyl radical abstracting an H atom from an adjacent PUFA, resulting in a lipid hydroperoxide (LOOH) and a second lipid radical (Figure 1.5). The hydroperoxy derivatives can undergo degradation, producing another free radical thus initiating further lipid peroxidation. The termination of these events occurs when the free radicals react with another free radical, protein, or compound that acts as a free radical trap, forming a stable non-radical end product (Figure 1.5) (Davies, 1996). $OH^{\cdot}$ is the most efficient biologically relevant oxygen free radical to attack PUFA, whereas $O_2^{\bullet-}$ is insufficiently reactive.

1. Initiation: $OH^{\bullet}$ abstracts an H (*hydrogen atom*) from one of the carbon atoms in the fatty acid chain forming a molecule of water and leaving the carbon atom with an unpaired electron (in red). The free radical formed reacts with O_2 to form peroxyl radical (Equation 1.6);

$$>CH + OH^{\bullet} \longrightarrow >C^{\bullet} + H_2O \qquad \text{[Equation 1.6]}$$

Figure 1.5: Series of reactions in lipid peroxidation



2. Propagation: This might then steal an H from a nearby side chain making it now a radical, propagating a chain reaction;

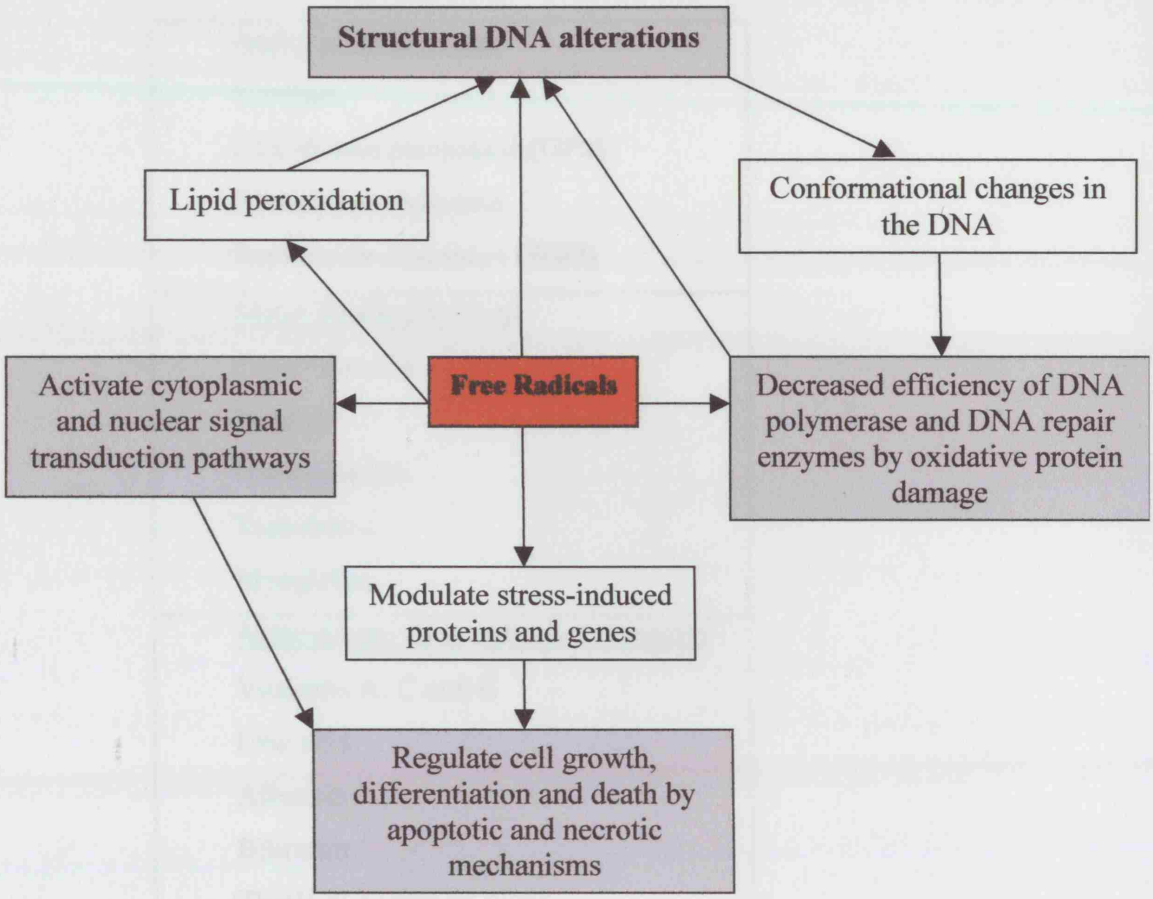
$$>C^{\bullet} + O_2 \longrightarrow >C-O-O^{\bullet} \qquad \text{[Equation 1.7]}$$

3. Termination: occurs when two radicals meet and each contributes its unpaired electron to form a covalent bond, producing non-radical products.

The free radical formation is terminated but with the result that the fatty acid side chains of membrane lipids may have been deformed as to damage the membrane. Lipid peroxidation is autocatalytic and consequently can amplify the damage produced by free radicals.

Oxygen free radicals may also react with nucleic acids, proteins, and carbohydrates though these biomolecules are less susceptible to attack than fatty acids. For example, due to their high electrophilicity and reactivity, and production near DNA, oxygen free radicals can add to DNA bases and abstract H atoms (Pryor, 1988). Sugar and base lesions are seen, the most common being 8-hydroxyguaninine, as well as strand breaks and cross-links (Wiseman and Halliwell, 1996). Interaction between these changes and the mechanisms of DNA synthesis and repair can result in structural alterations including base pair mutations, rearrangements, deletions and sequence amplifications, as well as gross chromosomal alterations, resulting in cell mutagenesis, carcinogenesis and cell death (Wiseman and Halliwell, 1996; Dizdar et al, 2003). DNA-protein cross-links can also occur which interfere with gene expression and DNA repair (Dizdar et al, 2003). In proteins, free radicals may oxidise aromatic amino acids and disulphide bonds (S-S), with serious implications for the integrity and function of various extracellular proteins including immunoglobulins, albumin and collagen. It is not surprising that oxygen free radicals and ROS have been shown to possess many characteristics of carcinogens (Wiseman and Halliwell, 1996; Figure 1.6).

Figure 1.6: **Potential carcinogenic characteristics of ROS**
(*Wiseman and Halliwell, 1996*)



1.4 Defences against Free Radical Damage

Many antioxidants in biological systems are thought to lower the risk of disease and delay the process of ageing. Antioxidant defence mechanisms include the inhibition of free radical production, scavenging of ROS/oxygen free radicals by antioxidants, enzyme-catalysed degradation, and processes which repair or remove damaged cell components such as lipid peroxides. Damage by ROS and free radicals is usually limited by endogenous antioxidants such as such as vitamins C, E and A, uric acid (Knapen et al, 1999) and free radical and ROS scavengers enzymes including

Table 1.1: Natural Antioxidant Defences

Antioxidant Enzymes
Catalase
Glutathione peroxidase (GPx)
Glutathione reductase
Superoxide dismutase (SOD)
Metal Binding Proteins
Ceruloplasmin
Ferritin
Haemoglobin
Transferrin
Myoglobin
Antioxidants (free radical scavengers)
Vitamins A, C and E
Uric acid
Albumin
Bilirubin
Thiols
Cystein
Carotenoids

Intracellular antioxidant activity is mainly attributable to enzymatic systems, but the antioxidant activity in plasma and follicular fluid is mostly derived from low molecular weight antioxidants, mainly uric acid, vitamins E and C and carotenoids (Ghiselli et al, 2000). An inverse relationship exists between TAC and oxygen free radical/ROS concentrations in biological samples, where the predominant antioxidant activity is non-enzymatic.

Both mitochondria and cytosol contain abundant enzymes that catalyse the breakdown of $O_2^{\bullet-}$ into H_2O_2 and O_2, and the detoxification of H_2O_2.

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \text{[Equation 1.8a]}$$

H_2O_2 is decomposed enzymatically by catalase and GPx:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \qquad \text{[Equation 1.8b]}$$

GPx, a selenium-containing enzyme, destroys peroxides before they can damage cell membranes, and is more efficient in dealing with low concentrations of H_2O_2 than catalase.

$$H_2O_2 + 2GSH \xrightarrow{\text{GPx}} 2H_2O + GSSG \qquad \text{[Equation 1.9]}$$

Haemoglobin, transferrin and myoglobin are antioxidants by virtue of their ability to bind metal ions, thus preventing their catalytic effects on free radical production. Antioxidant enzymes such as SOD, catalase and GPx also eliminate species ($O_2^{\bullet-}$ and H_2O_2) capable of initiating free radical chain reactions, while small molecule antioxidants such as vitamins C and E, urate and glutathione, are chain-breaking antioxidants able to 'repair' oxidizing radicals directly.

Vitamin E (α-tocopherol) is the major antioxidant in membranes while vitamin C (ascorbic acid) functions in aqueous compartments. Vitamin E is capable of interrupting chain reactions occurring in lipid peroxidation, breaking the covalent links that ROS have formed between fatty acid side chains in membrane lipids. It also scavenges free radicals generated by incomplete reduction of O_2 and by the normal

activity of some oxidative enzymes (Ehrenkranz, 1980; Buettner, 1993; Hamilton et al 2000). Vitamin E is particularly effective in preventing the attack of peroxides on unsaturated fatty acids in membrane lipids, being considerably more lipophilic than vitamin C (Van Acker, 1993). Vitamin C acts as an aqueous chain reaction-breaking antioxidant, scavenging a wide range of ROS, including $O_2^{\bullet -}$ and H_2O_2 (Beyer, 1994). It protects against peroxide-mediated oxidation of plasma low-density lipoprotein. Because of the high reducing potential of its carbon-carbon double bond, vitamin C readily donates one or two hydrogens and electrons to a variety of oxidants, including oxygen free radicals, peroxides, and $O_2^{\bullet -}$ (Buettner, 1993).

Vitamins E and C function together in a cyclic-type of reaction, where vitamin E is converted to a radical by donating a labile $H^{\bullet}$ to a lipid or lipid peroxyl radical (van Acker, 1993; Beyer, 1994; Hamilton et al, 2000). The oxidized vitamin E radical is relatively unreactive, and can be reduced to its original form by vitamin C at the surface of the cell membrane where both vitamins meet (van Acker, 1993) (Equation 1.9).

$$AscH^{-} + TO^{\bullet} \longrightarrow Asc^{\bullet -} + TOH \qquad \text{[Equation 1.9]}$$

AscH – *vitamin C* ; TO – *vitamin E*

Antioxidants have reduction potentials that place them in the middle of the 'pecking order' (Table 1.2). The location in this order provides them with enough reducing power to react with ROS (Buettner GR, 1993).

Table 1.2: The Pecking Order

Depending on their reduction potential, antioxidants can recycle each other.

For example, ascorbate with a reduction potential of +282 mV can recycle tocopherol ($TO^{\bullet}$; +480 mV) and urate$^{\bullet-}$ (+590 mV).

Buettner, 1993

Redox Couple	$E^{o\bullet}$/mV
$HO^{\bullet}, H^{+}/H_2O$	**+2310**
$RO^{\bullet}, H^{+}/ROH$ (aliphatic alkoxyl radical)	**+1600**
$ROO^{\bullet}, H^{+}/ROOH$ (alkyl peroxyl radical)	**+1000**
$GS^{\bullet}/GS^{-}$ (glutathione)	**+920**
$PUFA^{\bullet}, H^{+}/PUFA\text{-}H$ (^{bis}-allylic-H)	**+600**
$TO^{\bullet}, H^{+}/TOH$	**+480**
$H_2O_2, H^{+}/H_2O, HO^{\bullet}$	**+320**
$Asc^{\bullet-}, H^{+}/AscH^{-}$	**+282**
$CoQ^{\bullet-}, 2H^{+}/CoQH_2$	**+200**
Fe(III) EDTA/Fe(II) EDTA	**+120**
$CoQ/CoQ^{\bullet-}$	**-36**
$O_2/O_2^{\bullet-}$	**-160**
$RSSR/RSSR^{\bullet-}$ (GSSG)	**-1500**
H_2O/e^{-}	**-2870**

1.5 Biological Consequences of Oxygen Free Radical Reactions

Accumulated oxidative stress damage to lipids, proteins and mitochondrial and genomic DNA may lead to mutation, loss or inactivation of DNA, synthesis of abnormal proteins and changes in membrane lipid composition. Damage to DNA may involve direct oxidative process (e.g. by hydroxyl radicals), indirect damage by inappropriate binding of the end-products of lipid peroxidation, failure to repair DNA because of oxidative protein damage to polymerases and repair enzymes, and DNA cleavage by nucleases activated by rises in intracellular free Ca^{2+} (Halliwell and Gutteridge, 1999).

Membranes altered by peroxidation have modified fatty acid composition, disruption of permeability, decrease in electrical resistance and contain inactivated, cross-linked proteins (Richter, 1987). Lipid peroxidation and the products thereof are detrimental to cell viability and the collective effects of these cellular processes have been implicated in numerous pathological conditions (Davies, 1996), as discussed below. Free radicals are therefore implicated in a wide range of pathological processes, though often it has been difficult to demonstrate direct involvement.

1.5.1 *Role of ROS in Human Disorders*

Oxidative stress is mutagenic and potentially carcinogenic, and has also been linked with aging, diabetes mellitus, atherosclerosis and inflammatory disease (Halliwell and Gutteridge, 1999). Increased serum levels of lipid peroxides are associated with the angiopathy seen in diabetes mellitus (Sato et al, 1979) and atherosclerosis (Platcha et al, 1992). Numerous studies have concluded a pivotal role for ROS in degenerative and inflammatory conditions, post-radiation effects and aging (Halliwell and Gutteridge, 1999). Lewis et al (1995) found lower antioxidant levels in semen of infertile men with poor motility, and lower levels of molecular antioxidants in the presence of ROS. Furthermore, Besler and Comoglu (2003) found a marked decrease in plasma TAC in association with increased markers of lipid peroxidation during acute attacks of multiple sclerosis. These studies lend support to the concept that an inverse relationship exists between non-enzymatic antioxidants and ROS/oxygen free radicals.

Hyperphysiologic levels of oxygen are associated with clinical conditions such as neonatal bronchopulmonary dysplasia, and adult respiratory distress syndrome, suggesting an adverse influence of oxygen free radicals on airway epithelia (Southom

and Powis, 1988; Knight, 1998). Neonatal hyperoxia is widely linked to retinopathy of premature infants (Todd et al, 1998; Askie and Henderson-Smart, 2000). More careful controlled use of oxygen in the preterm newborn and administration of the lipid-soluble antioxidant vitamin E, have decreased the incidence of eye complications in the neonate (Raju et al, 1997).

Morris et al (1998) found no evidence of elevated circulating markers of oxidative stress in the plasma of preeclamptic women compared to women with clinically normal pregnancies. In their study, they compared plasma levels of 8 epi-prostaglandin $F_{2\alpha}$, lipid hydroperoxides, malondialdehyde and vitamin E among pregnant preeclamptic and non-preeclamptic women, as well as in non-pregnant women. Though these markers of oxidative stress were raised in normal and preeclamptic pregnant women (compared to non-pregnant individuals), there was no difference between the pregnant groups. This suggests that human pregnancy is accompanied by increased oxidative stress, which is not the cause of preeclampsia. In contrast, a placebo-controlled study (Chappell et al, 1999) on the effect of a combination of vitamins C and E supplementation (started between 16 and 22 weeks of pregnancy) on preeclampsia, found greater than 50% reduction in the incidence of preeclampsia in a high-risk population, defined by either a history of preeclampsia in previous pregnancy that required preterm delivery or abnormal doppler flow velocity (resistance index $\geq 95^{th}$ percentile for gestation or the presence of an early diastolic notch) at 20 weeks gestation.

In a further study authors Chappell et al (1999) looked at the effect of vitamins C and E supplementation on indices of oxidative stress in women at risk of preeclampsia, characterised as in previous study. They compared 'at risk' women taking antioxidant

supplements (vitamins C and E) with those taking placebo and a third group classified as 'low risk' which received no supplementation. They found that antioxidant supplementation in women 'at risk' resulted in improvement of biochemical indices of preeclampsia. These studies investigated plasma levels of markers of increased oxidative stress, whereas it is believed that the pathogenesis of preeclampsia is placenta-related, as suggested by the rapid resolution of preeclamptic symptoms after delivery. Looking at local placental levels of these indices may reveal evidence of placental oxidative stress. Also, in the latter study, women with a history of preeclampsia were classified as 'at risk', which may not necessarily be the case since this condition is more common in first pregnancies. Furthermore, it would be interesting to determine the effect of antioxidant supplementation in the 'low risk' group in the study of Chappell et al (2002) on the development of preeclampsia.

The role of enhanced placental $O_2^{\bullet-}$ generation leading to oxidative stress is increasingly recognised (Hubel, 1999). The onset of maternal blood flow in the placenta at 10 – 12 weeks of normal pregnancy, results in a local increase of oxygen tension and a parallel increase in placental expression and activity of antioxidant enzymes (Jauniaux et al, 2003). These authors postulated that a defective response to this oxidant stimulus could lead to trophoblast degeneration and impairment of the second wave of trophoblast invasion, which is associated with preeclampsia.

1.5.2 *Role of ROS in Male Fertility*

Although the generation of ROS and oxygen free radicals is normally associated with phagocytic leucocytes, mammalian spermatozoa were the first cell type in which this activity was described (Halliwell and Gutteridge, 1999). Under physiological

conditions oxygen radicals are produced by sperm through the leakage of electrons onto molecular oxygen from the mitochondrial electron transfer chain (Alvarez et al, 1987). In men, subfertility has been ascribed to excess production of free radicals in the semen that overwhelms available antioxidants. Mammalian spermatozoa are rich in PUFA which makes them susceptible to lipid peroxidation resulting in reduced motility, presumably because of a rapid loss of intracellular ATP through damaged cell membranes, causing axonemal damage (de Lamirande and Gagnon, 1995).

Many studies have implicated oxygen free radicals and ROS in both oligo- and astheno-zoospermia (Riley and Behrman, 1991; Zini et al, 1993; Aitken, 1995; Padron et al, 1997). Padron and colleagues (1997) compared ROS production between 24 men with spinal cord injury and 19 healthy control subjects. High levels of oxygen free radicals (up to 2,000 times) were found in sperm suspensions from men with spinal injuries relative to controls. Spinal injury patients had elevated seminal white blood cells counts compared with controls, and these were likely to be the source of the free radicals. Though the total sperm count did not differ between the two groups, the percentage of morphologically normal spermatozoa was lower in men with spinal injury. Furthermore, sperm motility in men with spinal injury was inversely related to the level of free radicals. Indeed the ratio of dead-to-live immotile sperm in men with spinal cord injury is twice that in normal men (Brackett et al, 1998). Suleiman et al (1996) also reported higher levels of lipid peroxides in sperm from infertile men with low sperm motility; indeed lipid peroxide levels were inversely related to sperm motility. The authors also found that vitamin E supplementation improved sperm motility and reduced malondialdehyde (MDA) concentrations in spermatozoa. Spermatozoa from infertile men also show nuclear

alterations in terms of abnormal chromatin, microdeletions, aneuploidy and DNA breaks (Gerardo et al, 2000).

Vitamins C and E and selenium are often used to treat male factor subfertility even though antioxidant levels do not appear to be reduced in otherwise normal sub-fertile men. The effectiveness of these treatments remains controversial (Kessopoulou et al, 1995; Tarin et al, 1994; Suleiman et al, 1996; Donnelly et al, 1999). Tarin et al (1994) randomly allocated and cultured human oocytes, spermatozoa and embryos from 83 couples to a group of 39 with 62.5 μM ascorbate supplementation in the culture medium and another group without supplementation. Ascorbate had no beneficial effect on fertilisation or embryo viability.

There are reports of beneficial effects of ROS on sperm function. Aitken et al (1989) reported increased sperm-zona interaction following induction of limited peroxidation in human sperm. Furthermore, Bize et al (1991) found that increased generation of H_2O_2 in hamster sperm preparations, by the addition of glucose oxidase, accelerated the onset of the acrosome reaction and capacitation. It therefore appears that a delicate pro-oxidant/antioxidant balance is required for optimal function of spermatozoa.

The association between oxygen free radicals/ROS and male infertility raises the possibility that these substances might also play a role in female reproduction. An imbalance in the pro-oxidant/antioxidant milieu existing in pre-ovulatory follicular fluid and elsewhere along the female genital tract, might be the final pathway by which some known and unknown aetiological factors influence fertility in women.

However, the roles of ROS and oxygen free radicals in female reproduction have been sparsely investigated compared to men.

1.5.3 *Role of ROS in Female Fertility*

It is possible that follicular fluid oxygen free radicals and ROS might have important roles in female reproduction, especially in couples with 'unexplained' infertility, as has been described in men. Studies on the effects of ROS, oxygen free radicals and antioxidants on female reproduction have tended to concentrate on evaluation of specific antioxidants, such as superoxide dismutase (SOD), vitamin C and GPx, as indirect measures of ROS activity (Miyazaki et al, 1991).

Miyazaki et al (1991) found ovulation *in vitro* to be markedly reduced when SOD was added to culture medium containing rabbit ovary explants, implying that $O_2^{\bullet-}$ is required for ovulation. In whole animal studies, Ho et al (1998) produced female mice lacking copper-zinc SOD by genetic manipulation. Female homozygous SOD knock-out mice showed markedly reduced fertility compared with wild-type and heterozygous SOD knock-out mice. Although the mice ovulated and conceived normally, they had a marked increase in embryo lethality (post-implantation embryo deaths). A similar experiment by Marzuk et al (1998) concurred with these results since it reported that the heterozygote SOD knock-out mice produced an average of 1.0 litter/month with 8.6 offspring/litter, whereas homozygote SOD knock-out mice produced an average of 0.23 litter/month with an average of 2.7 offspring/litter. These results imply that $O_2^{\bullet-}$ and other free radicals are detrimental to embryonic development at least when present at abnormally high concentration due to SOD disruption. This assertion is perhaps supported by studies on human embryos cultured

in microdroplets of medium containing either 20% or 5% O_2 concentration (Dumoulin et al, 1999). Culture under 5% O_2 resulted in a higher mean incidence of blastocyst formation per IVF cycle than in the 20% O_2 group, due to an abnormally low cell number in blastocysts from the latter group. Improved embryo development under conditions of reduced O_2 tension has been reported in mouse, sheep and cattle (Bavister, 1995).

It appears that successful interaction between the oocytes and spermatozoa requires optimal levels of ROS in human (Blondin et al, 1997), and that oxidative stress is a possible cause of defective embryo development (Agarwal et al, 2003). Jauniaux et al (2003) reported an association between early pregnancy loss and exposure of the embryo to high concentrations of oxygen, through early establishment of placental intervillous blood flow. This effect is more prevalent in the central regions of the placenta than the periphery. They also confirmed oxidative damage in samples of placenta obtained after miscarriage.

The studies in this thesis are different from many of the studies described above, in that TAC and antioxidant consumption in follicular fluid were related to the subsequent fate of the oocyte from the follicle, during *in vitro* fertilization (IVF) procedures. This essentially investigated the influences of follicular antioxidants and oxygen free radicals on oocyte preovulatory development and its subsequent competence.

Attaran et al (2000) determined follicular fluid free radical/ROS activity and total antioxidant capacity (TAC) in 53 women undergoing IVF, using a chemiluminescence technique described in Section 2.4.2. TAC levels were similar in

each group when outcome was considered. Women who became pregnant however had higher ROS levels than those who did not, leading to a suggestion that ROS levels might be a potential marker for predicting success in IVF patients. In contrast, Paszkowski et al (1995) reported a greater mean activity of the antioxidant enzyme GPx in follicles yielding oocytes that fertilised, compared with follicles yielding oocytes that failed to fertilise, suggesting an adverse role of ROS in human fertilisation. This assumes that GPx levels were higher because they had not been used up by excess ROS production, but the higher levels of this enzyme might have been induced by a prevailing oxidative stress. Though these studies seem to contradict one another, they are not truly comparable. While Attaran et al (2000) looked at follicular fluid ROS and TAC using pregnancy as the final outcome measure, Paszkwoski et al (1995) studied a specific antioxidant (GPx) as an indicator of ROS activity, and considered oocyte recovery and fertilisation capability as outcome measures. A firm statement on the role of ROS on female reproduction can therefore not be made on the basis of these studies.

In an attempt to further define the relationship between ROS and female fertility, Paszkowski et al (1996) determined TAC in culture medium from 66 single preimplantation embryos, and found an average TAC decline of 5.5% (0.9-41.7%) after 24 hours of incubation. The incubation of poor quality embryos was however associated with a higher TAC loss compared with better quality embryos. The authors concluded that increased ROS activity was associated with impaired human embryo development. It is possible however that membrane disturbance in poor quality embryos, or fragmentation, caused excess free radical generation, at least in the IVF setting. This suggestion is supported by the study of Yang and colleagues (1998) who reported higher H_2O_2 concentrations in fragmented embryos compared to

non-fragmented embryos and unfertilised oocytes. They determined H_2O_2 concentrations within 62 human oocytes and embryos using fluorescence imaging and transmission electron microscopy and an in-situ apoptosis detection kit to observe DNA fragmentation. A cause-effect relationship was impossible to establish since it was equally likely that a higher concentration of H_2O_2 led to the oocytes and embryos being of poor quality, or that their poor quality caused them to produce more H_2O_2. Yang et al (1998) also suggested the existence of a direct relationship between increased H_2O_2 concentrations and embryo fragmentation and apoptosis.

The available evidence regarding the role of ROS and oxygen free radicals in human female reproduction is therefore inconsistent and requires further research. Moreover, the therapeutic role of exogenous antioxidants in human reproduction remains controversial (Paszkowski et al, 1996; Kessopoulou, 1995).

1.6 Hypothesis

This thesis tests the hypothesis that follicular fluid oxygen free radicals, ROS and antioxidants influence female reproductive performance in the IVF setting.

1.6.1 *Aims of the Thesis*

1. To investigate the effects of oxygen free radical/ROS production in human follicular fluid on early reproductive outcome in the IVF setting, by determining follicular fluid total antioxidant capacity (TAC), as a marker of free radical/ROS activity, and correlating it with early reproductive outcomes.
2. To compare follicular fluid TAC between fertile and infertile women in natural cycles.

3. To investigate the possible interaction between follicular fluid TAC and etiological factors related to subfertility, as well as potential confounding variables such as smoking and age.
4. To investigate the protein content of follicular fluid and its relationship to total antioxidant levels (TAC) and early reproductive outcome.
5. To determine cytological correlates of follicular fluid antioxidant activity.

1.6.2 *Thesis Organisation*

Methodologies for the evaluation of free radicals and antioxidant activity are reviewed in Chapter 2, followed by a study to compare the Trolox Equivalent Antioxidant Capacity (TEAC) and the Ferric Reducing/Antioxidant Power (FRAP) assays, for measuring total antioxidant capacity (TAC) in follicular fluid. Good correlation between the two assays was demonstrated; the simpler FRAP assay was therefore used to measure TAC in further studies.

A study to evaluate the effect of oxygen free radicals, as indicated by follicular TAC and loss of antioxidants over 72 hr (antioxidant consumption), on early female reproductive outcome was then performed on a large number of follicular fluid samples. The interaction between TAC and TAC loss and aetiological factors related to subfertility, as well as potential confounding variables such as smoking and age, was also assessed. These results of these investigations are described in Chapter 3.

In Chapter 4, a review of the non-cellular composition of follicular fluid is presented. A study evaluating the correlation between human follicular fluid protein concentrations (as determined by Bradford assay) with TAC and early reproductive outcome is also described.

A review focused on the cellular components of ovarian follicular fluid, and the results of a study correlating follicular cell types with follicular fluid TAC and reproductive outcome are presented in Chapter 5.

A summary and conclusions from all the studies are presented in Chapter 6.

1.6.3 *Ethical Considerations*

Ethical approval was obtained from the Research Ethics Committee of Enfield and Haringey Health Authority before all these studies were carried out. The aims and nature of the investigations were explained to all subjects verbally and in writing. Subjects were assured that they were free to decline to participate in the study, or withdraw at any time, without prejudice to their clinical IVF treatment. Informed consent was obtained in writing from all the subjects.

Chapter 2

Measuring Free Radicals and Antioxidants

Outline

In this chapter, a summary is presented of the common methods used to evaluate free radical activity and antioxidant levels. This is followed by a comparative study of two assay methods used to determine total antioxidant capacity. The choice of assay used in the remaining studies is then discussed.

2.1 Assay methods for free radicals and ROS

Interest in the role of free radicals in physiological and pathological processes has led to the development of several techniques to measure these molecular entities. Direct estimation of free radical concentrations is difficult due to their reactivity and ultra-short half-lives (usually measured in microseconds). Indeed, free radicals produced *in vivo* react at or close to their source of formation, therefore methods devised to quantitate them are generally indirect. For example, the concentrations of end products of free radical reactions with lipid, protein and DNA within the body can be measured. Alternatively, free radical activity is often inferred from measurements of specific antioxidant concentrations or total antioxidant activity in biological fluids or tissues. Some of these methods are now described.

2.1.1 *Electron Spin Resonance (ESR) and Radical Trapping*

ESR spectrometry has been used to detect free radicals in human tissue samples and is the only technique that directly measures free radicals concentrations. By measuring the change in absorption of microwave energy within a continuously varying strong magnetic field, this method detects the number of "unpaired electron spins" in a sample. ESR is often used in conjunction with free radical trapping, in which an organic compound (the 'spin trap') is added to which the unpaired electron of free radicals transfers to form radical-adducts. The adducts are relatively stable

and can be detected in an ESR spectrophotometer. Two of the most commonly used spin traps are 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS). ESR is of limited value however because it is relatively insensitive and produces only semi-quantitative data (Cheeseman and Slater, 1993).

2.1.2 *Measurement of thiobarbituric acid and reacting substances (TBARS)*

The thiobarbituric acid (TBA) assay is the commonest method used to assess lipid peroxidation, as an indirect measure of free radical activity in biological samples. The assay is indirect and based on the reaction of 2 mol of TBA with 1 mol of malondialdehyde (MDA), one of the aldehyde products of lipid peroxidation. The biological sample is boiled with acidic TBA and the pink MDA-TBA complex can be extracted into an organic solvent, such as butanol, and quantified spectrophotometrically from its visible absorbance (max 532 nm) or by spectrofluorimetry (exc 532 nm, em 553 nm). This assay methodology is considered straightforward and sensitive, but its application to biological samples can be problematic (Janero, 1990). For example, the apparent "TBA reactivity" varies with the exact concentration and type of acid, and period of heating, used for MDA extraction (Gutteridge, 1982). Also, the iron content of reagents and the storage of samples at –70°C may affect the estimated concentrations (Yagi, 1984).

2.1.3 *Measurement of lipid hydroperoxides (LOOH)*

Lipid peroxidation occurs through a free radical chain reaction initiated by the abstraction of a hydrogen atom from a polyunsaturated fatty acid (PUFA) by a reactive free radical, followed by a sequence of propagative reactions (Holley and Cheeseman, 1993). LOOH are the major initial products of lipid peroxidation and can

be measured by a variety of techniques. They possess a diene structure with characteristic absorbance maxima at 234 nm. Measurement of this absorbance provides a useful index of peroxidation in pure lipid systems and in tissue preparations. It is also possible to measure plasma LOOH with commercially available assay kits. One such kit (Bioxytech LPO-560 Assay, Oregon, USA) relies upon the reaction of LOOH with a haem compound, concomitantly oxidising a precursor to produce methylene blue, which is measured spectrophotometrically.

Another indirect assay of free radicals/ROS is based on the ability of low concentrations of LOOH to activate the cyclooxygenase reaction, catalysed by prostaglandin endoperoxide synthase (Kulmacz et al, 1990). A polarographic oxygen electrode is used to monitor oxygen consumption by the reaction, providing an estimate of enzyme activity and thus LOOH concentration, and indirectly ROS/ free radical levels. LOOH can also be reduced with triphenylphosphine to hydroxy acids, which are subsequently estimated by gas chromatography-mass spectrometry (Hughes et al, 1986). Both these assay methods have two inherent problems. They provide only indirect measures of free radical/ROS activity, indeed of LOOH concentrations, and the equipment required to perform them is expensive to buy and maintain, and is unsuited to rapid sample throughout.

2.2 Assay methods for Antioxidants

Free radical-scavenging antioxidants delay or protect against oxidative damage produced by free radical reactions. Antioxidants are often measured as a surrogate for oxygen free radical activity, based on the assumption that antioxidant levels are inversely related to the free radical activity in a sample (Section 1.4, Chapter 1). Sometimes levels of specific antioxidants, such as ascorbate and GPx, can be

determined as indirect measures of free radical activity. Measuring specific antioxidants does not however truly represent the total antioxidant capacity within a sample, since it does not consider potential interactions between some of these antioxidants which are discussed below. Measurement of specific antioxidants cannot therefore give a true reflection of ROS/oxygen free radicals levels.

The total antioxidant capacity (TAC) in body fluids has been widely employed in studies on oxygen free radicals and ROS. TAC estimation assesses the cumulative action of all antioxidants in a sample, *i.e.* the capacity of known and unknown antioxidants and their synergistic interactions to protect against free radical and oxidative stress. An example of this synergism is that vitamin C may chemically regenerate the antioxidant capacity of vitamin E; likewise vitamin E may regenerate the reducing power of vitamin C (Packer et al, 1979; Stocker et al, 1986; Buettner, 1993). Estimates of TAC may therefore give more biologically relevant information regarding total antioxidant potential and oxygen free radical levels, than that obtained by measuring individual antioxidants, since it is more indicative of the total propensity of the sample to counter oxidative stress (Holley and Cheeseman, 1993). TAC considers the cumulative action of all the antioxidants present in the sample assayed, thus providing an integrated parameter, rather than being the simple sum of measurable antioxidants. TAC is normally measured as the molar equivalents of a given test solution to scavenge a free radical challenge, independent of the activity of any one antioxidant present in the mixture.

2.2.1 *The TRAP assay*

The total (peroxyl) radical-trapping antioxidant parameter (TRAP) assay is widely used to study antioxidants in biological fluids. This assay, originally developed by

Wayner et al (1985), is based on the property of "azo-initiators", such as 2,2'-azobis-(2-amidinopropane) hydrochloride (ABAP) and 2,2'-azobisisobutyronitrate (AIBN), to undergo thermal decomposition producing a peroxyl free radical flux at a constant (temperature-dependent) rate. The measurement of TRAP requires the determination of the time that the antioxidant activity in a sample is able to resist peroxidation by the peroxyl free radical flux. In this inhibition assay, antioxidant in the sample induces a lag phase and a 'signal' denotes when all antioxidants have been used up. The signal may be indicated by monitoring oxygen consumption in a thermostated oxygen electrode cell (Wayner, 1985), free radical induced degradation of a fluorophor (eg phycoerythrin) in a thermostated fluorescence cell (McKenna et al, 1991) or by chemiluminescence (Lissi et al, 1995; Said, 2003). Thus specialised 'signal' detection equipment is required, which is expensive and not available in many laboratories (Schofield and Braganza, 1996).

In the assay of Wayner et al (1985), the consumption of dissolved oxygen provides a measure of the lipid peroxidation rate, the consumption remaining low until all antioxidants are consumed. Oxygen consumption increases as unquenched azo-initiator free radicals induce the formation of lipid free radicals, which react with oxygen to produce lipid peroxyl radicals and LOOH. The 'lag phase' before the increase in oxygen consumption is proportional to the antioxidant capacity of the sample. The assay is often standardised against known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), an antioxidant vitamin E analogue. The method is cumbersome, time-consuming (up to 2 h per sample) and can be applied to only limited numbers of samples (Young, 2001). Another problem with the original TRAP assay was that the oxygen electrode tended to be unstable over the assay time required, causing the baseline to shift.

2.2.2 *The enhanced chemiluminescence assay*

This assay depends on a chemiluminescent reaction generating a steady light emission by the interaction of horseradish peroxidase, H_2O_2, and luminol, the latter reagent being the source of chemiluminescence (Said et al, 2003). The power of the antioxidants in a sample added to this assay reaction, to quench the free radical generated chemiluminescence, is compared to that of a standard solution of Trolox. The resulting TAC of the test sample is measured as molar Trolox equivalents. The chemiluminescence method is accurate, but cumbersome and time consuming, because fresh signal reagent must be prepared and standardized with Trolox each time the assay is performed (Said et al, 2003). Also, expensive equipment such as luminometer is needed to measure the chemiluminescence.

2.2.3 *The TEAC Assay*

In this thesis, a derivative of the TRAP method of TAC estimation was used, the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Rice-Evans and Miller, 1994) When 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is incubated with H_2O_2 and metmyoglobin, which has peroxidase activity, stable free radical cations ($ABTS^{\bullet+}$) are produced with a characteristic absorbance at 600 nm. The TEAC assay is based on the quenching of $ABTS^{\bullet+}$ by hydrogen-donating antioxidants present in a test sample added to this reaction. Thus the A_{600} nm remains low until hydrogen-donating antioxidants in the sample decline (Miller and Rice-Evans, 1996). The length of the lag phase and the reduction in the rates of $ABTS^{\bullet+}$ accumulation and A_{600} nm increase after the lag phase, are related to the antioxidant capacity of the sample. Assays are standardized using varying amounts of Trolox as a

comparator antioxidant to produce a standard curve, allowing a Trolox equivalent antioxidant capacity (TEAC) value to be calculated, i.e. the millimolar Trolox concentration having the equivalent total antioxidant capacity as a test sample (Rice-Evans and Miller, 1994). A major advantage of this method is that it utilises simple spectrophotometric detection and can be performed in most laboratories.

Some TEAC assay methods assess the generation of $ABTS^{\bullet+}$ by spectrophotometric analysis after a fixed time and the overall inhibitory effect of sample antioxidants on $ABTS^{\bullet+}$ accumulation is measured. Another method measures $A_{600\,nm}$ over time so as to define the length of the lag phase between reaction initiation and $ABTS^{\bullet+}$ accumulation. It has recently been suggested that the former methodology is less accurate, especially at shorter incubation times, since the Trolox-response standard curve linearity and variability improve with longer assay incubation time prior to spectrophotometric determination (Wang et al, 2004).

2.2.4 *The FRAP Assay*

A further assay of TAC was used in this thesis, the ferric reducing/antioxidant power (FRAP) assay (Benzie and Strain, 1999; Prior and Cao, 1999). The FRAP assay uses the reducing power of antioxidants in a sample in a redox-linked colorimetric method. At low pH, antioxidants in the sample induce reduction of a colourless ferric 2, 4, 6-tri-(2-pyridyl)-1,3,5-triazine (Fe^{3+}-TPTZ) complex to the ferrous form (Fe^{2+}-TPTZ), which has an intense blue colour and can be monitored by measuring the absorbance at 593 nm using a spectrophotometer. A standard curve is prepared by addition of known concentrations of ferrous ions to the assay (Benzie and Strain, 1996). The assay essentially determines the "antioxidant power" in the sample, as

mole equivalents of ferrous ions producing the same absorbance on the standard curve.

Most antioxidants assays measure the ability of antioxidants to inhibit the oxidative effects of reactive species purposely generated in the reaction mixture. The FRAP assay however measures the total antioxidant power in the sample, assessed by its ability to act as a reductant towards ferric ions. FRAP values for adult plasma range between 600 - 1650 μmol/liter with a mean of 1017 μmol/liter (Benzie and Strain, 1996; Benzie and Strain, 1999). It should be noted that only antioxidants which are able to reduce ferric ions to ferrous ions, are quantified in this assay.

2.2.5 *Comparing the TEAC and FRAP Assays*

The main antioxidant contributors to TAC estimated by TEAC assay are uric acid and albumin, while the main contributors by FRAP assay are uric acid and ascorbic acid (Cao and Prior, 1998). Indeed the component of TAC attributable to specific antioxidants varies with assay method, as then will TAC estimates for the same sample. Certainly, protein-associated antioxidant activity derived from cysteine residues is unlikely to be estimated using the FRAP assay, but is included in TEAC estimates.

The FRAP and TEAC assay methodologies have not been applied to ovarian follicular fluid; assessment of their performance when used to estimate TAC in this body fluid is one goal of this thesis. Correlation between TEAC and FRAP TAC estimates in a novel body fluid can not be assumed, as the degree of correlation seems to depend on the biological sample under investigation (Cao and Prior, 1998; Wang et al, 2004)

The FRAP and TEAC assay methodologies have been used to measure TAC in plasma and have been extensively evaluated for analysis of serum TAC. Relative to the FRAP assay, the TEAC assay tends to underestimate serum TAC, probably because sample dilution has non-linear effects on TAC estimates. Furthermore, as discussed in Section 2.2.2, $ABTS^{\bullet+}$ absorbance is estimated after a fixed, sometime very short (3 mins), incubation in some TEAC assay formats. The results of such studies are inherently inaccurate since the variability and non-linearity of the Trolox standard curve are considerable (Wang et al, 2004). Longer fixed incubation times of ca. 30 min, or determination of the length of lag phase, should improve accuracy and comparability with other methods of TAC estimation. This was the case when the TEAC assay was compared with the oxygen radical absorbance capacity (ORAC) assay, which requires a fluorescent detector and takes more than 60 min longer to complete than the FRAP or TEAC assay (Cao and Prior, 1998).

The accurate assessment of oxidative stress in biological systems is a problem for all investigating the pathological roles of free radicals and ROS. Numerous assays have been described to measure antioxidant status and various products of free radical damage; the plethora of available techniques attests to the fact that no ideal method is available. A problem with available assays for TAC is that they poorly correlate with each other because various antioxidants react differently in each assay. This is, in part, due to the different oxidative insult applied within the assay. It is therefore important to understand the relative contribution of individual antioxidants to TAC estimated using a particular methodology. Due to these problems, TAC estimates should always be discussed in context with their assay conditions and methodology.

TAC estimates from different studies should only be compared when the conditions and methods by which they were measured are comparable or, better still, identical.

2.3 Estimation of follicular fluid TAC using TEAC and FRAP assays

2.3.1 *Objective of study*

This pilot study compared the TEAC and FRAP assay methodologies, which are widely employed for determining TAC in biological samples, to find an ideal assay to determine human ovarian follicular fluid TAC. It was performed to assess whether the easier and quicker FRAP assay could be used, instead of the TEAC assay, in the larger studies described later in the thesis.

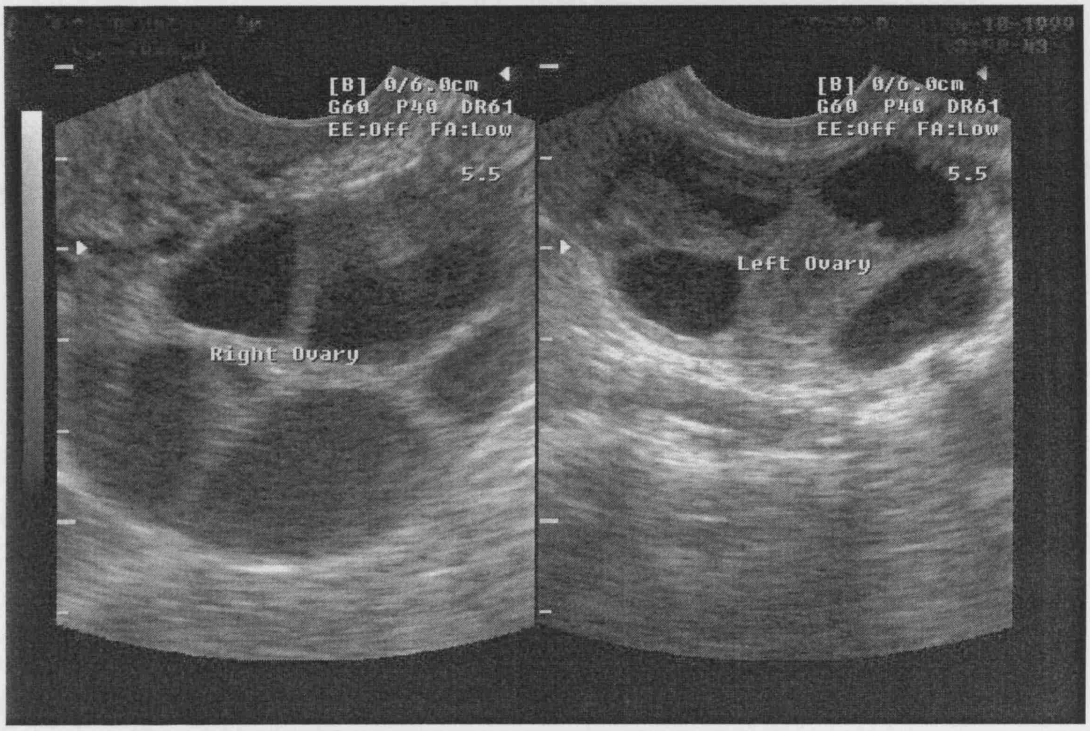
2.3.2 *Subjects and Methods*

This pilot study was carried out between November 2000 and December 2002. Ethical approval was obtained from the Enfield and Haringey Local research Ethics Committee (Number 300; 21.06.1999). Patients undergoing *in-vitro* fertilization (IVF) and embryo transfer at the London Female and Male Fertility Centre, Highgate Private Hospital, London were approached to participate, and their informed consent was obtained. Women who were on antioxidant vitamins, hormonal contraception or other hormonal preparations were excluded.

A long protocol for ovulation induction was used. Patients were first treated with daily 300 μg subcutaneous injections of buserelin (Hoechst UK Limited, Hounslow, UK), a GnRH agonist, from day 21 of the menstrual cycle until the ovarian follicles matured in the next cycle (average 18 – 20 mm diameter). Daily subcutaneous gonadotrophin injections (recombinant FSH; Puregon from Organon Ltd, Newhouse, Lanarkshire, UK) were commenced on the third day of the subsequent cycle, also

until follicles were mature. The dose of FSH varied between 50 and 150 mg daily depending on patients' responses. Ovarian responses were monitored with serial transvaginal ultrasound scanning and serum oestradiol estimation, as necessary. Human chorionic gonadotrophin (10,000 units) (Profasi; Serono, Feltham, UK) was administered when the leading follicle reached a diameter of 18 - 20 mm on ultrasound (Figure 2.1).

Figure 2.1: Trans-vaginal ultrasound scan of ovaries prior to oocyte recovery. Note the multiple large diameter follicles present in each ovary. Scale bars are spaced at 1 cm.



Oocyte recovery was performed 36 - 40 h after hCG administration, under ultrasound guidance by the vaginal route. A 16-gauge double lumen ovum pick-up needle (Cook Ireland Ltd, Limerick, Ireland) connected to a regulated vacuum pump (model KMAR-5000; Cook Ireland Ltd) at -230 mmHg, was used to separately aspirate each

follicle. Oocytes were transferred immediately to culture medium from the follicular fluid samples, which were processed as described below. Mature oocytes were inseminated with spermatozoa 2 – 6 h after retrieval, and fertilisation assessed microscopically 16 - 24 h after insemination. Two or three days after oocyte retrieval, 2 or 3 embryos were replaced in the uterine cavity, depending upon patient age, embryo quality, number of previous attempts and the couple's wishes.

Immediately after removal of the oocyte, the follicular fluid was centrifuged at 600 g for 5 min to remove cellular components. The supernatant was stored at –80°C for no longer than one week until the TAC was analysed. Antioxidant activity in plasma is stable for up to 6 months at this temperature (Miller and Rice-Evans, 1996; Valkonen and Kuusi, 1997; Comstock et al, 1995). Only follicular fluid with insignificant contamination with blood or culture medium was used in these studies.

TAC was determined in 13 follicular fluid samples using the TEAC and the FRAP assays, as described below, to ascertain the degree of correlation between TAC estimates provided by the two assay methodologies. To evaluate the reproducibility of the FRAP assay, 10 further samples were also assayed in triplicates using this assay.

2.3.2.1 *The TEAC Assay*

The TEAC assay was performed by the method of Rice-Evans and Miller (1994).

Reagents

Phosphate buffered saline (PBS), pH 7.4

5 mM ABTS (Sigma-Aldrich, Dorset, UK)

50 μM methaemoglobin (Sigma-Aldrich, Dorset, UK)

3 mM hydrogen peroxide (H_2O_2) (Sigma-Aldrich, Dorset, UK)

2.5 mM Trolox in PBS (Sigma-Aldrich, Dorset, UK)

Protocol

50 μl of follicular fluid was added to a premix containing 750 μl PBS, 100 μl of 5 mM ABTS and 100 μl of 50 μM methaemoglobin in a semi-micro plastic cuvette. 50 μl of 3 mM hydrogen peroxide was added and the absorbance at 723 nm ($A_{723\ nm}$) was monitored at 25°C in a recording spectrophotometer (Perkin Elmer λ5, Beaconsfield, UK). The length of the lag phase until the formation of $ABTS^{\bullet+}$ and increase in $A_{723\ nm}$, was taken as a measure of TEAC. The reaction was calibrated using aliquots of Trolox, prepared directly as 2.5 mM in PBS.

2.3.2.2 *The FRAP Assay*

The FRAP assay was performed by the method of Benzie and Strain (1996).

Reagents

40 mM HCl

300 mM sodium acetate buffer (pH 3.6); 3.1 g Na-acetate.$3H_2O$ (Sigma-Aldrich, Dorset. UK) and 16 ml glacial acetic acid (Sigma-Aldrich, Dorset. UK) made up to 1,000 ml with distilled H_2O.

10 mM TPTZ in 40 mM HCL (3.1 mg/ml; Sigma-Aldrich, Dorset, UK)

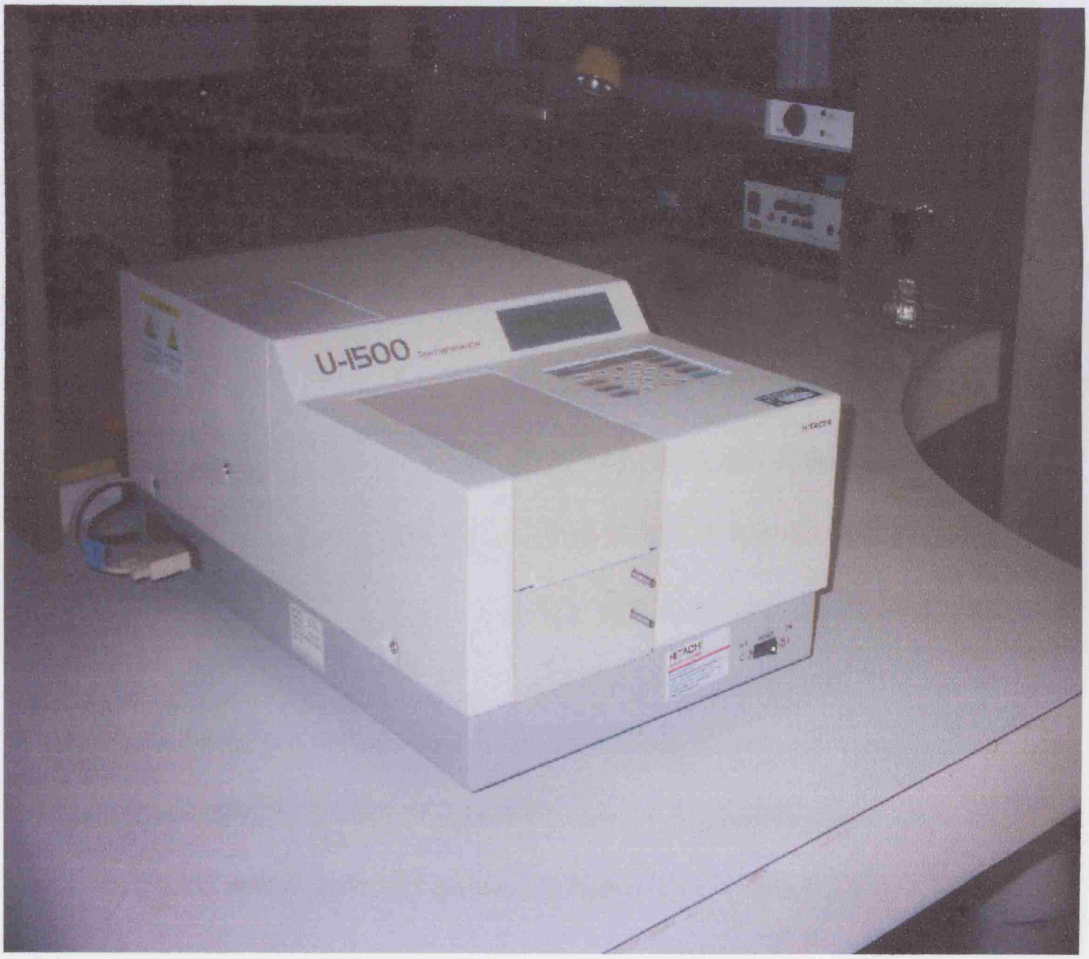
20 mM $FeCl_3.6H_2O$ (5.4 mg/ml in distilled H_2O; Sigma-Aldrich, Dorset, UK)

1 mM $FeSO_4.7H_2O$ (0.176 mg/ml) was diluted in distilled water to provide 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1 mM $FeSO_4$ standards

Protocol

10 mM TPTZ, 20 mM $FeCl_3$ and 300 mM acetate buffer, pH 3.6, were mixed together in a ratio of 1:1:10, respectively, to give the working FRAP reagent. This was freshly prepared as it was not stable for more than 24 hours. 50 µl of follicular fluid was added to 1 ml of FRAP reagent in a semi-micro plastic cuvette and the absorbance at 593 nm was measured after 10 min in a spectrophotometer (U-1500, Hitachi. Wokingham, UK; Figure 2.2). All measurements were taken at room temperature with samples protected from direct sunlight.

Figure 2.2 Spectrophotometer (Model U-1500; Hitachi. Wokingham, UK)



The FRAP assay was standardised using 0 – 1 mM $FeSO_4$, added at 50 µl per 1 ml of FRAP reagent. The $A_{593\ nm}$ vs Fe^{2+} standard curve was linear for absorbances between 0 and 0.8 (approx. 0 – 0.8 mM Fe^{2+}), with a slope of 19,500 $M^{-1}cm^{-1}$, as previously noted (Benzie and Strain, 1999). The TAC of a sample was taken as being equal to the Fe^{2+} concentration giving the same A_{593} nm. It should be noted that this would correspond to twice the concentration of either ascorbate or urate in the follicular fluid, since both are able to reduce two moles of Fe^{3+} per mole of antioxidant.

2.3.2.3 *Statistics*

Pearson linear correlation analysis was performed using SPSS for Windows, version 11, to compare the two methods. P values < 0.05 were considered significant.

2.3.3 *Results*

The TEAC and FRAP assays performed as expected when used to analyse TAC in 13 follicular fluid samples from IVF patients (Table 2.1). The mean follicular fluid TAC ± SD was 719 ± 140 µM by TEAC assay, and 537 ± 92 µM by FRAP assay. There was an inter-sample coefficient of variation (CV) of approximately 20%, presumably due, in the most part, to biological variability related to patient diet, pathology, age and other variables including those associated with lifestyle.

A significant linear correlation was observed between the results from the two assay methods ($r^2 = 0.96$; $P < 0.0001$; Figure 2.3), albeit the gradient of the regression line was 1.46, TEAC assays generally producing higher TAC estimates than FRAP assay.

Table 2.1: TAC estimates by FRAP (as μM Fe^{2+}) and TEAC (as μM Trolox) assays in 13 samples of follicular fluid derived from patients treated by IVF

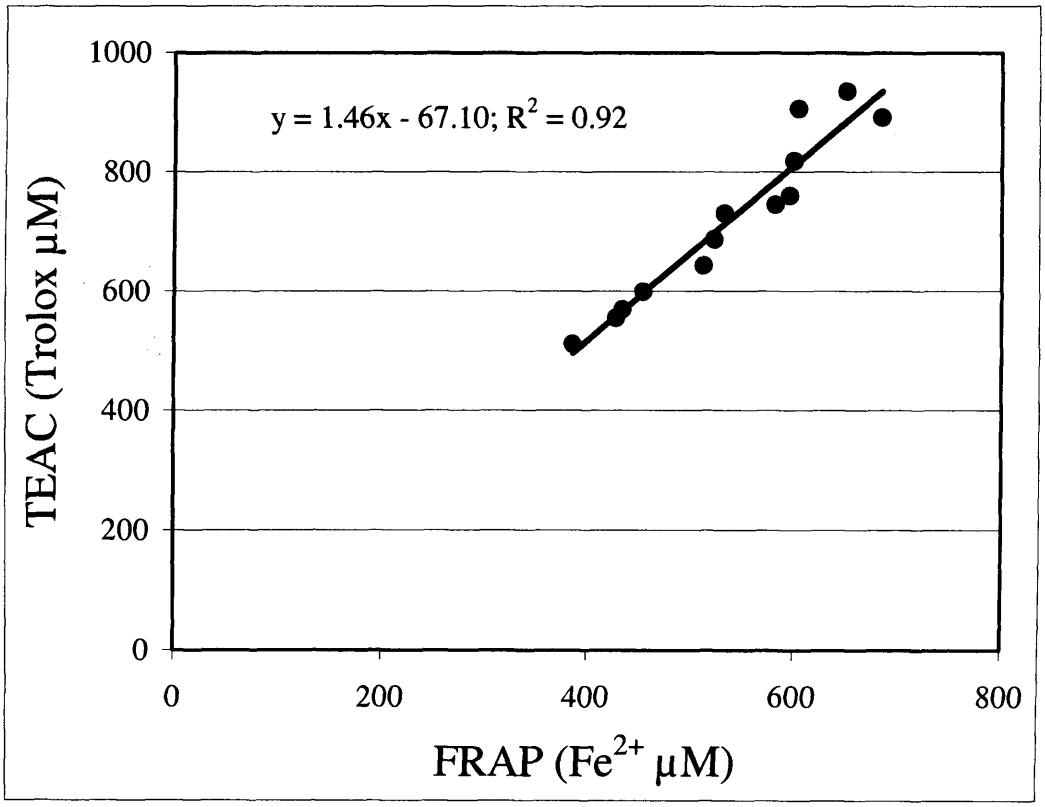
Samples	FRAP	TEAC
L3AB	427	555
R1AB	522	686
L2AB	684	891
R1AG	433	569
L1AG	650	934
R4AR	532	730
L1AR	599	818
R6AR	595	759
R2AR	385	511
L2AR	603	905
R15AR	581	745
L6AR	453	599
L12AR	512	642

When a further 10 follicular fluid samples were analysed in triplicate using the FRAP method, the mean CV within triplicates was 4.0% (Table 2.2), while the inter-sample CV was 10.5%. Analysis of variance showed that the inter-sample differences were not due to sampling errors ($F = 14.89$: $P = 7.7 \times 10^{-7}$); i.e. the probability that the variation between samples was due to sampling errors was less than 1 in a million.

Table 2.2: FRAP assay of follicular fluid samples in triplicates

Samples	1st	2nd	3rd	Mean	SD
1	626	631	610	622	11.0
2	593	637	619	616	22.1
3	701	669	689	686	16.2
4	549	564	553	555	7.8
5	713	689	634	679	40.5
6	741	669	697	702	36.3
7	673	687	674	678	7.8
8	521	589	556	555	34.0
9	596	523	567	562	36.8
10	692		751	722	41.7
Mean	**640.5**	**628.7**	**635.0**	**637.8**	**25.4**
SD	**74.4**	**58.4**	**66.8**	**64.0**	**13.9**

Figure 2.3: Correlation of TEAC and FRAP assay results



2.4 Discussion

The FRAP assay for measuring TAC has been validated for several biological samples, notably human serum and tears (Benzie and Strain, 1999; Choy et al, 2000). This method measures the capacity of the antioxidants contained in the analysed solution to reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ, which absorbs light at 593 nm. The TEAC assay, on the other hand, measures the ability of antioxidants in a sample, added to a reaction generating a stable free radical cation ($ABTS^{\bullet+}$) at constant rate, to quench free radical accumulation. In the TEAC assay used in this study, the delay in detection of the increase in A_{723} nm resulting from accumulation of $ABTS^{\bullet+}$, i.e. the 'lag phase', was used as an indicator of antioxidant activity (Schlesier et al, 2002). This study is the first application of either assay methodology to human ovarian follicular fluid.

An excellent correlation between the results of the TEAC and the FRAP assays was observed. Pellegrini et al (2003) also reported an excellent correlation between the results of the FRAP and TEAC assays, when evaluating TAC in various vegetables ($r = 0.977$, $P < 0.0001$) and beverages ($r = 0.997$, $P < 0.0001$). They also found that TEAC antioxidant estimates are well correlated with those from the TRAP assay ($r = 0.993$, $P < 0.0001$). In contrast, Prior and Cao (1999) did not find such a good correlation between serum TAC estimates derived using TEAC and FRAP assays. They attributed the poor correlation to inaccurate TEAC estimates, resulting from the use of inhibition percentage at a fixed time (rather than length of lag phase) in their assay methodology, and the fact that serum dilution had a non-linear effect on TEAC values. Indeed, the dilution of serum produced up to a 15% increase in the absolute TEAC estimates but not the FRAP estimates.

In the present study it was observed that the TEAC assay consistently gave higher estimates of TAC than the FRAP assay, and the gradient of the TEAC vs FRAP regression was 1.46. A dilution effect was not operating since follicular fluid was used undiluted, as it gave TAC estimates in the quantitative range in both assays. On a mole per mole basis, Trolox, α-tocopherol and uric acid have double the power of Fe^{2+} to generate the Fe^{2+}-TPTZ chromogen in the FRAP assay (Benzie and Strain, 1996). Thus it might be expected that FRAP estimates would be double those of the TEAC assay, and the regression line gradient should be 0.5. This erroneously assumes however that each antioxidant in a sample is equally reactive towards $ABTS^{\bullet+}$ or Fe^{3+}in the two assays, and/or that Trolox is an effective control for differences in antioxidant reactivity between assays; both assumptions are untrue. The apparent trend for TEAC values to be greater than FRAP values, may occur because the FRAP assay was relatively unreactive to some TEAC-reactive antioxidants. For example, the FRAP assay suffers from the disadvantage that it does not completely estimate the sulphydryl group-containing antioxidants (Cao and Prior, 1998), certainly not after 10 min incubations (Benzie and Strain, 1996). A 'background' was also noted in the FRAP assay, as the regression intercept was -67; *i.e.* when the TEAC value was zero the FRAP was 46 μM. The statistical relevance of this deviation from zero is questionable however.

For the purpose of the present studies, it was necessary to use a robust technique that would provide the basis for comparison between a large numbers of samples measured on different occasions. The decision to assess the FRAP assay was based on reports suggesting that it was quicker, simpler and cheaper to perform (Benzie and

Strain, 1999). The present study confirmed the simplicity, reproducibility and reliability of the FRAP assay making it a useful assay for future investigations. The observed mean intra-sample CV (for triplicates) of 4.0%, compares well with previous CV estimates of 3 and 10% (Pellegrini et al, 2003), and indicates the FRAP assay is consistent and reproducible.

The FRAP assay also had the advantage that many samples could be processed quickly and simultaneously. Comparable methods, including the TEAC assay, are more expensive and time-consuming and often require specialised equipment. The chemiluminescence-enhanced TRAP method, an alternative to the original oxygen electrode-based TRAP assay (Wayner et al, 1985), utilises a free radical sensitised chemiluminescent system measured in a LKB Wallac luminometer. The assay is considered reliable, but a maximum of only 24 samples could be processed in 8 hours (Said et al, 2003). This was considered too low for the purposes of the present study. Also, the required luminometer costs approximately £20,000, compared with £4,000 for the simple spectrophotometer needed for the FRAP assay.

It is well documented that the measured antioxidant capacity in a sample depends on which technology and free radical generator or oxidant is used in the assay (Pellegrini et al, 2003). A comparison of the TEAC and FRAP assay methods using follicular fluid, allowed the FRAP assay to be selected as the most convenient, reliable and reproducible method. The results of the FRAP assay compared favourably with those of the TEAC assay and the FRAP assay was simpler, quicker and relatively cheaper. It was therefore chosen for measuring follicular fluid TAC in the rest of the studies and can be recommended for use in future similar studies.

Publication from this work

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Chapter 3

Follicular Fluid Antioxidants and Early Reproductive Outcome

Outline

TAC and antioxidant consumption (TAC loss over 72 h) were determined using the FRAP assay, as measures of free radical activity, in follicular fluid samples from patients undergoing IVF (Study A) and from fertile and sub-fertile women undergoing laparoscopy during normal menstrual cycles (Study B). The influence of these parameters on early human female reproductive outcome and fertility is discussed.

3.1 Introduction

The possibility that oxygen free radicals and ROS have roles in human reproduction was suggested nearly 60 years ago (MacLeod, 1943). Spermatozoa are rendered dysfunctional by lipid peroxidation, which alters membrane function and increases cell permeability to electrolytes, resulting in metabolic impairment and disrupted morphology, motility and fertility (Cummins et al, 1994). Human spermatozoa primarily produce $O_2^{\bullet-}$, which is then broken down to generate H_2O_2 (Equation 3.1), thought to be the most toxic oxidising species acting on spermatozoa (Griveau and le Lannou, 1997).

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{[Equation 3.1]}$$

The effects of free radicals and ROS on female fertility have received little attention, but evidence exists for both physiological and pathological effects. Ho et al (1998) and Marzuk et al (1998) reported that SOD knock-out female mice exhibited reduced fertility potential and/or increased embryonic lethality, linking $O_2^{\bullet-}$ free radicals with poor reproductive performance. GPx concentrations are greater in follicular fluid

from follicles yielding fertilisation-competent oocytes, than from follicles yielding fertilisation-incompetent oocytes, suggesting that ROS have an adverse effect on the oocyte (Paszkowski et al, 1995). Likewise, the incubation of poor quality embryos is associated with a higher TAC loss in the culture medium compared with better quality embryos (Paszkowski et al, 1996), suggesting that excessive ROS/free radical production is related to defective embryo development, at least in vitro. Indeed, Yang et al (1998) found a higher H_2O_2 concentration in fragmented embryos, than in non-fragmented embryos and unfertilised oocytes.

Free radicals and ROS have also been proposed to have beneficial effects in human female reproduction. Attaran et al (2000) found that women who became pregnant following IVF treatment, had higher ROS levels in follicular fluid than did women who failed to become pregnant. Similarly, SOD activity is higher in human follicular fluid from follicles that yielded fertilisation-incompetent, rather than fertilisation-competent, oocytes (Sabatini et al, 1999). Generation of ROS for 24 hr in culture medium during bovine oocyte *in vitro* maturation improved subsequent oocyte/embryo developmental competence (Blondin et al, 1997). This also suggests a beneficial role for ROS in oocyte maturation, albeit the relevance of this observation to human oocyte development is open to question.

That free radicals and ROS should be seen to have positive and negative effects on oocyte and early embryonic development is not surprising for several reasons. Firstly, many of the studies are not comparable because of the different markers of free radicals and ROS evaluated. The use of specific enzymes or antioxidants such as SOD, ascorbate and GPx as markers does not truly represent total antioxidant capacity, nor does it take into consideration the synergistic interactions that can occur

between antioxidant species. Studies investigating TAC might overcome these shortcomings. A further reason for these contrasting results is that some studies focused on follicular fluid TAC while others were conducted on culture medium. The final outcome measures also differed and a cause-effect relationship was impossible to establish in many studies. For instance, in the study by Yang et al (1998), it was equally likely that a higher concentration of H_2O_2 existed in the oocytes and embryos leading to their poor quality, as it was that the poor quality was the cause of greater production of H_2O_2. Similar consideration applies to the interpretation of the study of Paszkowski and Clarke (1996), where greater antioxidant consumption in the culture medium was associated with poor quality pre-implantation embryos. All that can be said is that a relationship exists between H_2O_2 concentration, antioxidant consumption and embryo quality. Nevertheless, this information may be useful in the selection of embryos for transfer.

It is possible that free radicals and ROS are permissive for female reproductive success in 'normal' concentrations, but higher or lower concentrations are detrimental. The use of different models, outcome measures, end-points and assay methodologies of limited comparability in this field of research would tend to produce the present situation in which different studies give conflicting results.

The work in this chapter was performed to test the hypothesis that oxygen free radicals and ROS in follicular fluid influence oocyte development and subsequent early reproductive outcome in the IVF setting. It also investigated whether the activity of oxygen free radicals and ROS in follicular fluid was related to the aetiology of infertility in women undergoing IVF. These studies used follicular fluid TAC, and the change in follicular fluid TAC over a 72 hr incubation (TAC loss), both

assessed using the FRAP assay, as markers of oxygen free radical and ROS generation.

Study A investigated follicular fluid TAC in relation to early reproductive outcome, aetiology of infertility and confounders such as smoking and age, in a group of sub-fertile women undergoing IVF. All of these women were treated with hormonal preparations to induce ovulation, as described in Section 2.3.2. This treatment may have influenced follicular fluid TAC, therefore in a Study B, follicular fluid TAC was compared between fertile (control) and sub-fertile women during their natural menstrual cycles. Studies A and B were designed to achieve Aims 1 and 2 of this thesis, respectively (Section 1.6.1). There has been no previous study of follicular fluid TAC as a marker of ROS and oxygen free radical concentrations in non-stimulated women undergoing normal menstrual cycles.

3.2 Materials and methods

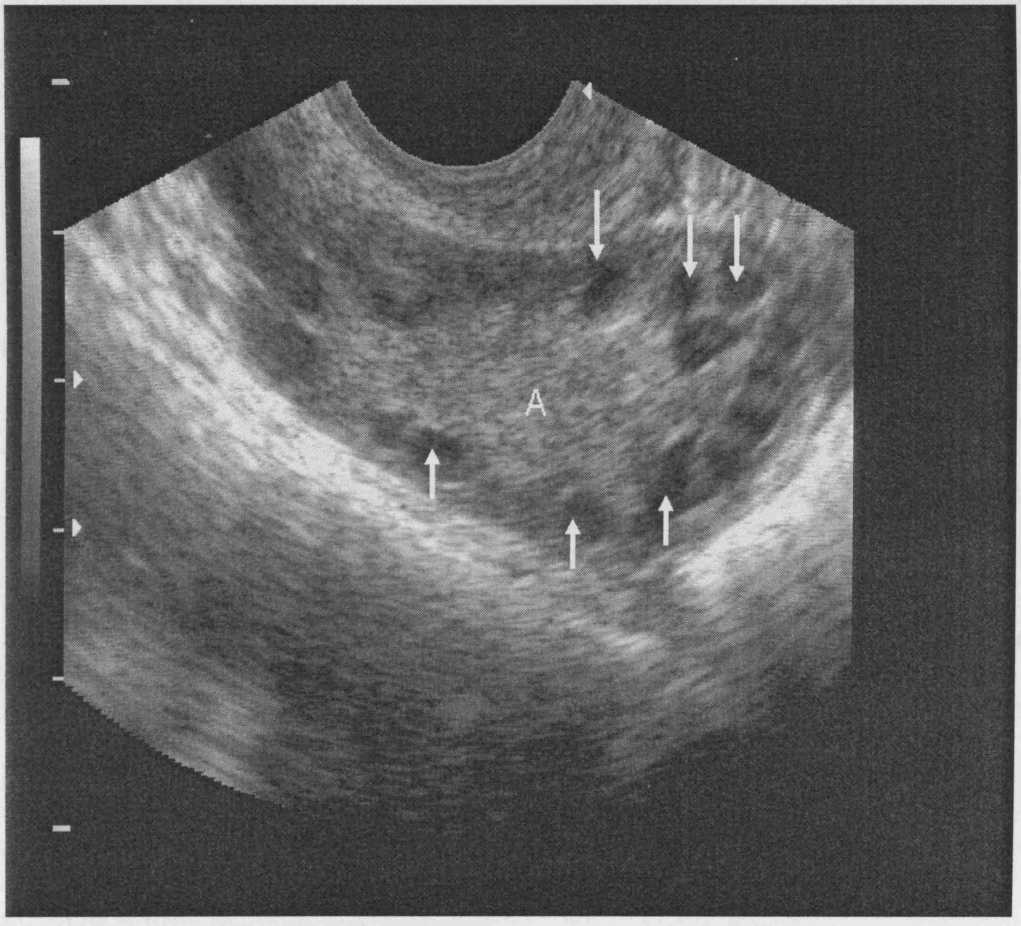
3.2.1 *Study A: The effect of follicular fluid TAC on reproductive outcome in women undergoing IVF*

This study was carried out between December 1999 and December 2002. Follicular fluid samples were obtained from the London Female and Male Fertility Centre, Highgate Private Hospital, London, and assays conducted in the Obstetrics and Gynaecology Departmental Laboratory at the Royal Free Hospital, London. Subjects were women undergoing controlled ovarian hyperstimulation as a component of IVF treatment for infertility. They were approached to participate in the study, provided with a study description and their informed consent obtained. Relevant patient information was collected on a standard questionnaire during consultation, which was

kept with their clinical notes. Women who were on antioxidant vitamins, hormonal contraception or other hormonal preparations were excluded.

All patients were investigated by transvaginal ultrasound, before ovulation induction, for the presence of polycystic ovary (PCO) morphology. This was defined as the presence of 10 or more peripherally distributed follicles of diameter 2 to 8 mm, associated with a hyperechoic ovarian stroma (Figure 3.1).

Figure 3.1: Transvaginal ultrasound scan of a polycystic ovary showing a hyperechoic stroma (A) and peripherally distributed multiple follicles (examples marked with white arrows). Scale bars on the left are spaced at 1 cm



Normal ovarian morphology was defined as the presence of less than 10 follicles, but with at least one follicle of diameter greater than 8 mm. The definition of PCO morphology is consistent with that of Adams et al (1985), albeit they used transabdominal ultrasound. Furthermore, Saxton et al (1990) found a perfect correlation between ultrasound assessment and histological examination, when used to assess the number and distribution of cysts in ovaries with PCO morphology. The definition of PCO morphology in this thesis is slightly different from the new definition which requires that the number of follicles (2 - 9 mm in diameter) seen on transvaginal scan is $\geq$12 (Rotterdam PCOS Consensus Workshop, 2004). Furthermore, women with PCO morphology in this thesis do not necessarily have polycystic ovary syndrome (PCOS) as no single diagnostic criterion is sufficient for clinical diagnosis PCOS. PCOS is now considered to be a syndrome of ovarian dysfunction, with the primary features of hyperandrogenism and PCO morphology (Rotterdam PCOS Consensus Workshop, 2004).

Ovulation induction, follicular tracking, oocyte recovery and subsequent oocyte and follicular fluid processing were carried out as described in Section 2.3.2. Although the oocytes from each patient were pooled at the time of fertilisation, each oocyte was located within an area marked on the under surface of the petri dish. This allowed oocyte identification up to the time of embryo transfer, so that early reproductive outcome could be compared with follicular fluid TAC in the source follicle for each oocyte. The volume of follicular fluid obtained was carefully measured once the oocyte has been removed and follicular fluid contaminated with blood or culture medium was excluded

Follicular fluid TAC was determined using the FRAP assay, which was employed for the reasons detailed in Section 2.4. Follicular fluid samples were thawed to room temperature, then assayed as described in Section 2.3.2.2. Samples and assays were kept in the dark as much as possible and protected from direct sunlight when not in a dark box. Each follicular fluid sample was assayed a second time after 72 h, having been kept in the dark at room temperature between the assays. The TAC estimates at 0 and 72 h provide a measure of the ability of antioxidants in the sample to resist stress due to free radicals and ROS at those times. This "two-point assay" was also used because the on-going production of free radicals and ROS in the follicular fluid over the 72 hr incubation, resulting from the presence of haem groups and other catalytic factors for free radical and ROS production, provided oxidative stress. This challenged the TAC of the sample to quench it, hence TAC estimates were less after 72 h. The decrease in TAC after 72 h was thought to give a measure of the pro-oxidant generating activity in a follicular fluid sample, as well as the ability of antioxidants in the sample to combat the free radicals and ROS produced. The 72 h incubation time was selected in preliminary experiments described in Section 3.4.

The mean baseline TAC values and the TAC loss after 72 h were correlated with the presence or absence of an oocyte in the individual follicles, and the subsequent fate of the oocytes, i.e. whether fertilisation-competent or not, and whether the resulting embryos were viable or not after 72 h culture (Figure 3.2 and 3.3). The volume of follicular fluid obtained during oocyte retrieval was also correlated with these early reproductive outcome measures. Fertilisation-competence was assessed microscopically to determine whether oocyte cleavage was initiated. Viability was also assessed microscopically, after 72 h, viable embryos exhibited uniform cell morphology, intact nuclei and cytoplasm, and were at the 8 cell stage (Figure 3.2).

Non-viable embryos exhibited evidence of nuclear fragmentation, non-uniform cell morphology and cytoplasmic fragmentation and vacuolation (Figure 3.3).

Figure 3.2: Viable 8-cell stage embryo after 72 h

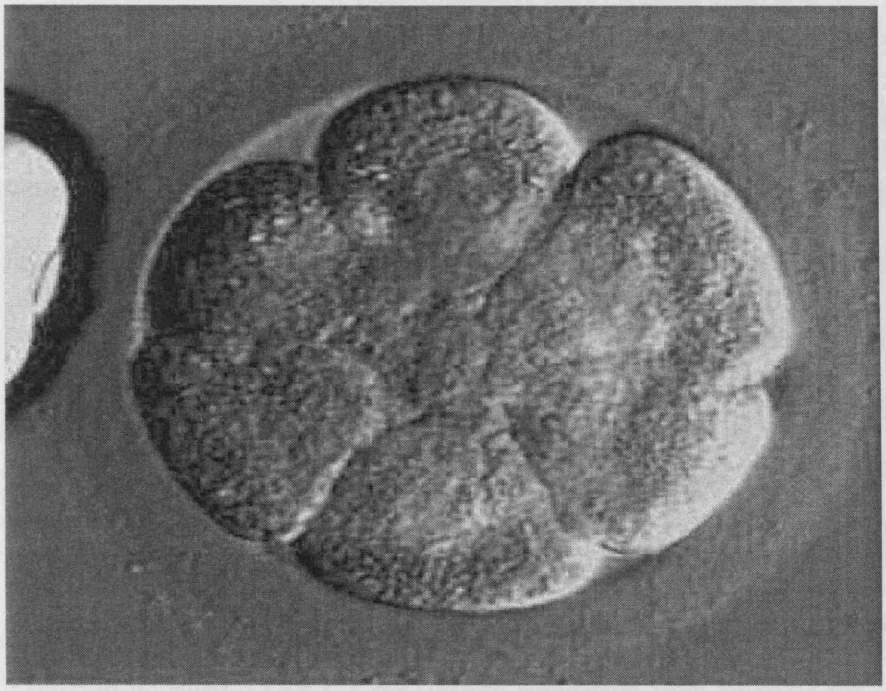
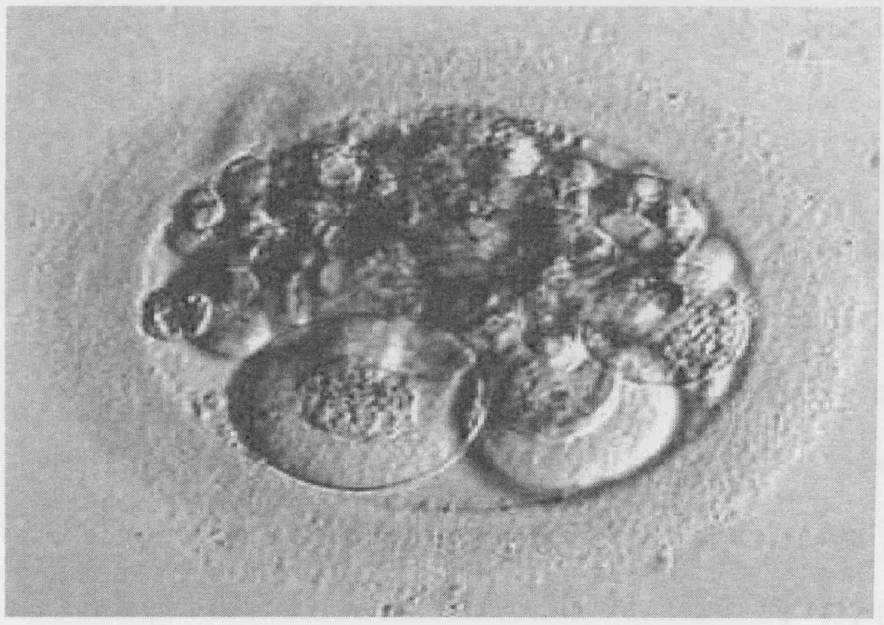


Figure 3.3: Non-viable embryo after 72 hr; note extensive fragmentation



12 μm

3.2.2 *Study B: Comparison of follicular fluid TAC in Graafian follicles from fertile and sub-fertile women during normal menstrual cycles*

This study was carried out between December 1999 and December 2002. Follicular fluid samples were obtained from women undergoing laparoscopy at the North Middlesex Hospital, London, and assays conducted in the Obstetrics and Gynaecology Departmental Laboratory at the Royal Free Hospital, London. The women were recruited from the general gynaecological clinic and required diagnostic laparoscopy either for investigation of sub-fertility or laparoscopic sterilisation. The objectives of the study were explained to the subjects verbally and by way of written information leaflets; similar information was also sent to their general practitioners. Written consent was obtained for needle aspiration of follicular fluid during the laparoscopic procedure. Women who were on antioxidant vitamins or hormonal preparations were excluded. In addition, women with irregular menstrual cycles were excluded because it was not possible to accurately time laparoscopy to the immediate preovulatory period.

Of necessity, laparoscopy was timed to coincide with the immediate pre-ovulatory period, calculated on the basis of the individual's last menstrual period, so as to harvest follicular fluid from mature Graafian follicles. Patients on the waiting list were requested to call on the first day of their menstrual period so that surgery could be scheduled during the next pre-ovulatory period (between Day 11 and 13 of menstrual cycle in women with 28 day cycles). When this was not possible, further attempts were made in subsequent cycles. Because of the practical problems involved, including availability of operating theatre space, it was not possible to recruit a large number of women and the results of a pilot study are presented.

Subjects fasted overnight and were admitted for surgery the following morning. The risks involved in the procedure were again explained, including the possibility that a laparotomy might become necessary to deal with complications arising during laparoscopy. All subjects were fit for surgery from the anaesthetic point of view and full blood counts were confirmed to be normal. At laparoscopy under general anaesthesia, the ovary containing the mature Graafian follicle was identified. The ovarian ligament was held with a pair of tissue forceps to keep the ovary steady while the follicle was aspirated with an Abbocath needle (Abbott Laboratories Ltd. Maidenhead, UK) attached to a syringe. Immediately after aspiration, follicular fluid was processed as described in Section 2.3.2, and the required surgery was completed.

Follicular fluid samples were defrosted to room temperature then TAC was evaluated using the FRAP assay at baseline and after 72 h at room temperature, as discussed in Sections 2.3.2.2 and 3.2.1. Mean follicular fluid TAC and TAC loss over 72 h were compared between fertile and sub-fertile women.

3.3 Statistics

3.3.1 *Determination of sample size*

A pilot study was performed using the FRAP assay to measure TAC in 107 follicular fluid samples, to allow estimation of the inter-sample variance (CV = 23%). Normal distribution of the data was confirmed using the Kolmogorov-Smirnov Test (*http://bardeen.physics. csbsju.edu/stats/KS-test.n.plot_form.html*).

The program G.Power 2 for Macintosh was used to perform a Power analysis, using the 2-tailed 't' test setting (Dupont and Plummer, 1998). This provided the number of samples required to test, with a certain Power, the null hypothesis that the means of

two independent sets of normally distributed data, of defined homogenous variance, are equivalent at a defined level of significance ($P < 0.05$). The 2-tailed test allows for differences in means in either direction. Initially, the number of samples was calculated to provide a Power of 0.8 of detecting a 10% difference in the mean TAC of two different groups, each with a CV of 23%, at $P < 0.05$. A control *versus* experimental sample ratio of 2:1 was assumed and the 10% difference in means was selected as being a likely physiologically relevant TAC difference related to lifestyle characteristics or the aetiology of infertility. This Power analysis indicated that sample sets of 128 and 64 (total = 192) satisfied the statistical conditions applied. For some patient lifestyle characteristics (e.g. having a smoking habit), it was unlikely that a sample group of 64 could be achieved. Therefore more follicular fluid samples were collected – 303 in all. Power analysis indicated that this sample group of 303 could be split 252-51 to achieve a Power of 0.8, 229-75 to achieve a Power of 0.9, and 193-110 to achieve a Power of 0.95, to detect a 10% difference in the mean TAC of the two different groups, with a CV of 23%, at $P < 0.05$.

3.3.2 *Analysis of main data*

All analyses were performed using the program Statistical Package for the Social Sciences (SPSS), version 11. The significance of differences in case frequencies in different aetiology and/or outcome groups were estimated using Pearson's Chi squared analysis. Homogeneity of variance between different aetiology and/or outcome data groups was investigated using the Levene statistic, which generates a probability (P) value for the likelihood that the null hypothesis, that variances between groups are equal, is true.

The significance of differences between data group means was initially investigated using analysis of variance (ANOVA). Homogeneity of variance was tested because it is a basic requirement of ANOVA and some post hoc analyses that variance is homogeneous between groups. Bonferroni's test was used for post hoc analysis when variance was homogeneous, this test being similar to Student's 't' test, except it accounts for the multiple pairwise comparisons made between group means when means of >2 groups are analysed, albeit it is considered more useful when the number of comparisons is limited – as in this study. In the few cases when variance between data groups was non-homogeneous, Dunnett's T3 test was used, which is suitable in this situation. Correlation analyses in this thesis were performed using the Pearson correlation test for data that are normally distributed. When the paired data were not normally distributed, the Spearman rank correlation, a non-parametric statistical method was applied.

In the vast majority of cases, the results reported in this thesis are the group means ± standard deviation, also with the group sample size (n). Two-tailed P values are shown and P values < 0.05 (highlighted in bold in the tables) are accepted as indicating a statistically significant difference between groups means, while those between 0.05 and 0.1 might indicate a trend worthy of further examination.

3.4 Results

3.4.1 *Optimising the time lag in the 2-point FRAP assay*

The aim of this experiment was to determine the stability of TAC estimates when follicular fluid was stored at room temperature in the dark, and whether the endogenous generation of free radicals and ROS under these conditions could be estimated by the change in TAC with incubation time. In a pilot experiment, 7

follicular fluid samples from the same patient were thawed to room temperature. TAC was assayed using the FRAP assay (t = 0) as described in Section 2.3.2.2, and again after 22, 46, 71 and 95.5 hours. Follicular fluid samples were kept at room temperature between assays, and the assays were kept in the dark as much as possible, protected from direct sunlight when not in a dark box.

A graph of TAC versus incubation time is shown in Figure 3.4. TAC declined in all samples during the incubation at room temperature. It was decided that an incubation time of 72 h should be used for the "two-point assay", as this time was practically useful - assays could fit into a working week – and also was long enough that TAC decline was substantial and could be accurately determined.

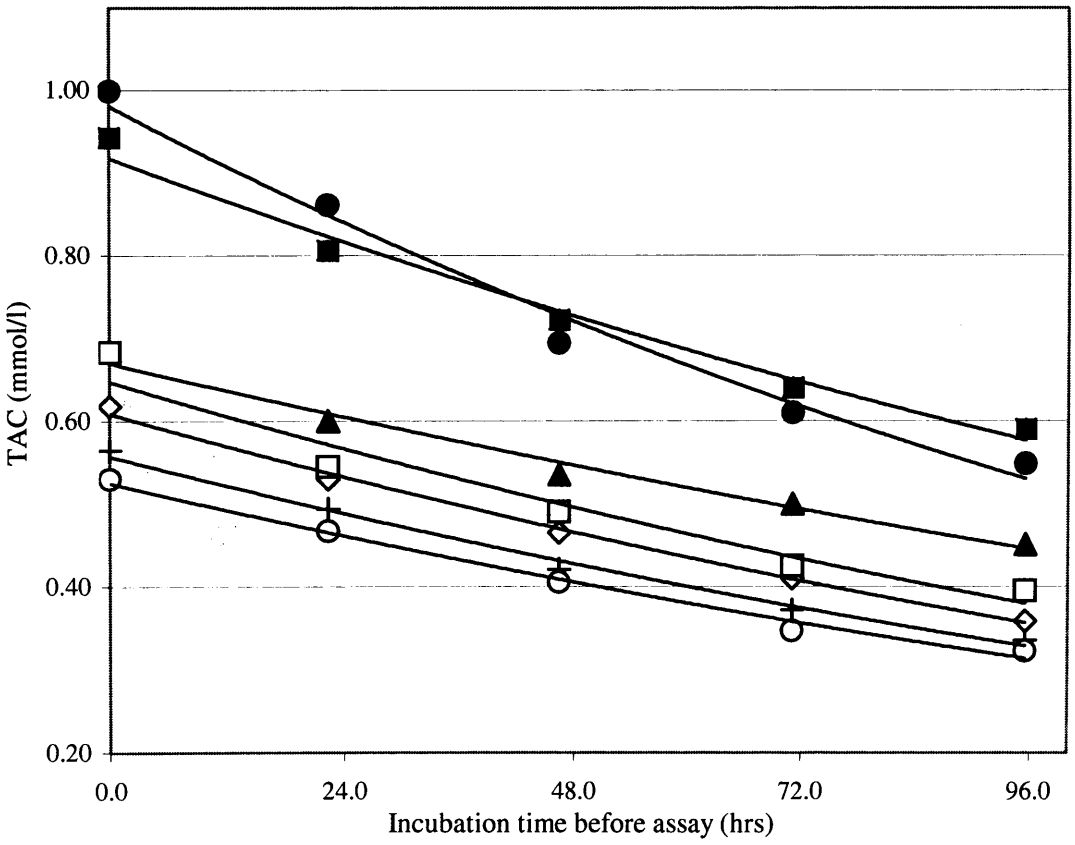


Figure 3.4: The change in TAC estimated by FRAP assay in 7 follicular fluid samples stored for varying periods at room temperature in the dark

As stated above (Section 3.2.1), the difference between TAC estimates at 0 and 72 h provides a measure of the innate ability of the antioxidants in the follicular fluid sample to resist stress due to free radicals and ROS, and also provides an indication of the pro-oxidant activity in the sample.

3.4.2 *Follicular fluid TAC in patients undergoing IVF (Study A)*

Follicular fluid aspirates (n = 303) were collected from 34 women during oocyte recovery for IVF treatment, as described in Section 3.2.1. Some of these women went through IVF treatment cycles more than once. Almost 72% of the follicles harvested (i.e. 218 of 303 follicles) yielded oocytes, of which 169 (77.5%) were successfully fertilised. Of these embryos, 134 (79.3%) survived and remained in good condition for 72 h until the day of transfer. The mean age of the subjects was 33.7 ± 4.7 years. The aetiological factors relating to patient infertility are shown in Table 3.1, the proportion of these factors being similar to those reported from other units in the Western world (Hull, 1996; Reproductive Health Outlook, 2002; Adamson and Baker, 2003).

Aetiology	N° of patients/ samples	As % total patients/ samples	Mean age (years)	N° >37 years patients/ samples	N° of smokers patients/ samples
Male factor	13/122	38/40.3	33.3 ± 4.0	1/8	1/1
Tubal factor	7/59	21/19.5	33.0 ± 6.1	2/17	0
Endometriosis	1/8	3/2.6	37.0	0	0
Endometriosis + tubal factor	1/10	3/3.3	32.0	0	1/10
Unexplained	12/104	35/34.3	34.0 ± 6.0	5/50	1/11
TOTAL	34/303			8/75	3/22

Table 3.1: Frequency of aetiological and confounding factors related to sub-fertility in women undergoi

TAC was estimated in all 303 follicular fluid samples using the two-point FRAP assay as described in Sections 3.2.1 and 2.3.2.2. Follicular fluid mean TAC and TAC loss in individual follicles (n = 303) were related to reproductive outcomes for the associated oocyte, allowing determination of the effect of follicular antioxidant environment on the oocytes. In all samples at baseline (i.e. t = 0), the mean TAC in follicular fluid was 0.680 ± 0.16 mmol/l, the 24% coefficient of variation (CV) representing the impact of many biological variables on this parameter, some investigated in this thesis.

3.4.2.1 *Relationship between individual follicular TAC and TAC loss and early reproductive outcome (follicle-by-follicle analysis)*

When TAC data for each follicle (n = 303) was related to early reproductive outcomes of IVF treatment, interesting differences emerged (Table 3.2). The TAC of follicular fluid from follicles containing oocytes was comparable with that from empty follicles. The mean TAC in follicular fluid from follicles that yielded oocytes that subsequently failed to fertilise was significantly lower, by 8%, than the mean TAC from follicular fluid associated with fertilisation-competent oocytes. Conversely, the mean TAC of fluid from follicles, the oocytes from which gave rise to non-viable embryos at 72 h, was significantly greater, by 8.6%, than the mean TAC in follicular fluid associated with oocytes that gave rise to 72 h viable embryos. The mean CV for the intra-individual inter-follicular TAC levels remained large (17.5%) but was less than the inter-patient CV of 24%. The optimal follicular fluid TAC for successful early reproductive outcome (i.e. oocyte+, fertilisation+, viability+) was therefore in the region of 0.680 mmol/l. Lower follicular fluid TAC

levels were associated with fertilisation incompetence, while higher levels were permissive for fertilisation, but were associated with embryo non-viability.

Outcome		N	Mean Baseline TAC (mmol/l)	95% Confidence Interval of the Difference Lower	Upper	P
Total		303	0.680 ± 0.16			
Oocyte retrieval	Yes	218	0.679 ± 0.16	-0.042	0.040	0.95
	No	85	0.680 ± 0.18			
Fertilisation	Yes	169	0.693 ± 0.16	-0.105	-0.005	**0.03**
	No	49	0.637 ± 0.15			
Embryo viability	Yes	34	0.680 ± 0.16	0.001	0.117	**0.04**
	No	35	0.739 ± 0.14			

Table 3.2: Mean baseline TAC in follicular fluid in relation to oocyte retrieval and subsequent fate

Follicular fluid TAC loss after 72 h (Table 3.3) was not influenced by the presence of an oocyte in the follicle, by the fertilisation competence of the recovered oocyte, or

Outcome		N	Mean TAC Loss (mmol/l)	95% Confidence Interval of the Difference Lower	Upper	P
Total		303	0.123 ± 0.084			
Oocyte retrieval	Yes	218	0.120 ± 0.083	-0.034	0.009	0.24
	No	85	0.132 ± 0.087			
Fertilisation	Yes	169	0.118 ± 0.085	-0.036	0.017	0.48
	No	49	0.127 ± 0.079			
Embryo viability	Yes	134	0.120 ± 0.087	-0.018	0.046	0.38
	No	35	0.106 ± 0.076			

Table 3.3: Follicular fluid TAC loss after 72 h in relation to oocyte retrieval and subsequent fate

by subsequent embryo viability. For the total 303 data set, the mean TAC loss was 0.123 ± 0.084 mmol/l.

Low follicular fluid volume was associated with subsequent oocyte fertilisation failure (Table 3.4). This may be because follicular fluid volume increases with the developmental maturity of the follicle and the oocyte it contains. Low follicular fluid volumes may therefore reflect oocyte immaturity. Follicular fluid volume had no influence however on oocyte recovery and embryo viability.

Outcome		**N**	**Mean Follicular Fluid Volume (ml)**	**95% Confidence Interval of the Difference**		**P**
				Lower	**Upper**	
Total		303	6.07 ± 2.34			
Oocyte retrieval	Yes	218	6.15 ± 2.37	-0.87	0.33	0.38
	No	85	5.88 ± 2.27			
Fertilisation	Yes	169	6.32 ± 2.34	-1.52	-0.02	**0.05**
	No	49	5.55 ± 2.38			
Embryo viability	Yes	134	6.29 ± 1.82	-0.79	0.70	0.91
	No	35	6.33 ± 2.46			

Table 3.4: Follicular fluid volume and reproductive outcome

3.4.2.2 *Relationship between TAC and TAC loss of all follicular fluid samples from individual women and reproductive outcome (subject-by-subject analysis)*

Follicles and oocytes in the same women develop in micro-environments which may vary but will also share common characteristics; the oocytes also share common genetic characteristics. TAC data was therefore further analysed on a patient-by-

patient basis (n = 34), with an expectation that clinically important differences between women might be highlighted.

Mean TAC and TAC loss in all follicular fluid samples obtained from individual women were calculated and related to their early reproductive performance. Based on the percentages of successful outcome when data for the individual 303 samples were examined, 72%, 78% and 79% cut-offs were selected to differentiate between subjects with 'poor' and 'good' oocyte recovery, oocyte fertilisation and embryo viability, respectively. These cut-off figures represent the prevalence rate of positive outcomes associated with the 303 follicular fluid samples; it is reasonable to consider that individuals exhibiting positive outcome prevalence higher than these cut-offs are 'good performers' and vice versa. Mean individual TAC and TAC loss were also compared between women who became pregnant and those who did not.

Correlation/regression analysis was also used to investigate the relationship between subject (n = 34) reproductive outcome rates and the mean subject-averaged follicular fluid TAC and TAC loss. This approach complimented the subject-by-subject approach using good/bad reproductive outcome setting, since it provides an indication of the linear continuity of any putative relationships, as well as an indication of the degree of interaction between follicular TAC and reproductive outcomes.

Analysis by comparison of mean follicular TAC and TAC loss for each subject (n = 34) showed similar changes (Table 3.5) as the follicle-by-follicle analysis, except that subjects in whom oocyte recovery was poor (<72%) exhibited a mean follicular TAC

8.6% less than did subjects in whom oocyte recovery was good (≥72%), albeit this result was of marginal significance.

Outcome		N	Mean TAC (mmol/l)	P	Mean TAC Loss (mmol/l)	P
Oocyte Recovery	<72%	16	0.669 ± 0.163	0.06	0.126 ± 0.095	0.35
	≥72%	18	0.732 ± 0.155		0.120 ± 0.075	
Fertilisation	<78%	13	0.650 ± 0.160	**0.03**	0.134 ± 0.094	0.16
	≥78%	20	0.730 ± 0.154		0.117 ± 0.080	
Viable Embryos	<79%	16	0.737 ± 0.146	**0.04**	0.117 ± 0.084	0.39
	≥79%	15	0.663 ± 0.170		0.122 ± 0.085	

Table 3.5: Subject mean follicular fluid TAC and TAC loss in relation to poor/good early reproductive performance in 34 IVF patients

In agreement with the follicle-by-follicle analysis however were the observations that mean follicular TAC in individuals with a poor fertilisation rate (<78%), was 11% lower than in those with a good fertilisation rate (≥78%), and the mean follicular TAC in individuals with <79% viable embryos at 72 h was 11.2% greater than in those with ≥79% viable embryos. There was no significant relationship between mean TAC loss in follicular fluid from the 34 women and early reproductive outcome (Table 3.5), also as noted in the follicle-by-follicle analysis.

The comparison of mean individual TAC and TAC loss were between women who became pregnant and those who did not showed a trend, albeit non-significant, for mean follicular TAC to be lower in women who achieved pregnancy, than in those who did not (0.634 ± 0.100 mmol/l vs 0.724 ± 0.130 mmol/l, respectively; P = 0.097), but there was no significant difference in mean follicular TAC loss (0.131 ± 0.059 mmol/l vs 0.126 ± 0.040 mmol/l, respectively; P = 0.82).

When correlation analysis was applied to the total subject set, subject-averaged follicular TAC was not correlated with oocyte recovery rate or oocyte fertilisation rate, but it was correlated with embryo viability. Likewise, subject-averaged follicular TAC loss was not correlated with oocyte recovery or embryo viability rates, but was correlated with oocyte fertilisation rate.

Success rate embryo viability (%) = {-120[subject-averaged TAC (mmol/l)]} + 156

$r = -0.476$; $P < 0.007$.

Oocyte fertilisation rate (%) = {-225[subject-averaged TAC loss (mmol/l)]} + 106

$r = -0.400$; $P < 0.021$.

The correlation/regression approach confirms therefore that the relationship between follicular TAC and embryo viability noted in the follicle-by-follicle analysis (i.e non-viability embryos had higher follicular TAC) is continuous and linear, and that approx 25% of the variability in embryo viability rates may be accounted for by variability in subject-averaged follicular TAC. This significant statistical association does not however confirm causation, indeed, no correlation was noted between fertilisation competence rate and subject-averaged follicular TAC, even though the follicle-by-follicle analysis suggested that fertilisation-incompetent oocytes were associated with low follicular TAC levels. Likewise, subject-averaged follicular TAC loss was correlated with oocyte fertilisation rate, yet no interaction between these parameters was apparent in the follicle-by-follicle analysis.

3.4.3 *Influence of the aetiology of infertility, age, PCO morphology and smoking on follicular fluid antioxidants and early reproductive outcome*

The aetiology of infertility can be clearly identified among many affected couples, and may include tubal blockage, anovulation or problems with the male partner (male factor). In other couples however the aetiology remains unknown, termed 'unexplained', despite thorough clinical investigation. Several confounding factors such as PCO, smoking and age are thought to influence fertility and may be important in cases of unexplained infertility. The effects of known aetiological factors on fertility, as well as the effects of some confounding factors, may be mediated through prooxidant-antioxidant imbalance in follicular fluid.

The relationships between the mean baseline TAC and TAC loss, and reproductive outcome, described in Section 3.4.2, do not take aetiology and confounding factors into account. This may explain the relatively large variances noted for these parameters in most sample groups (CVs of 24% for baseline TAC). To determine whether the influence of aetiology of infertility and confounding factors affect fertility through changes in follicular fluid antioxidants, their effect on baseline TAC, TAC loss and early reproductive outcome was investigated using uni- and multivariate ANOVA statistical analysis, within the total data set of 303 follicular fluid samples.

3.4.3.1 *Effects of the aetiology of infertility*

The interaction between the three major aetiological sets – i.e. tubal blockage (Tubal factor), male factor (MF) and 'unexplained', follicular fluid TAC and TAC loss and early reproductive outcome are depicted in Tables 3.6 A-C. These 3 aetiological groups comprised 94.1% of the total set of 303 follicular fluid samples studied. Two

other aetiological sets (endometriosis, n = 8, and endometriosis + tubal, n = 10) were omitted from this analysis due to their sample sizes being insufficient to ensure valid statistical analysis.

The complex tables depicting the influence of aetiology show two 'P' value columns. The Outcome 'P' values relate to the significance of the effect of reproductive outcome alone (i.e. oocyte+/-; fertilisation+/-; embryo viability+/-), on the mean TAC and TAC loss within each aetiological set. 'P' values under MF *vs* 'Others' relate to the effect of aetiology, at common reproductive outcome (e.g. oocyte^{+}, fertilisation^{-} etc), on follicular fluid TAC and TAC loss. The MF group consisted of women who had no obvious abnormality but whose partners had semen abnormalities. It was therefore taken as a 'control' group, with which the other two aetiology groups (tubal factor and 'unexplained') were compared, since no pathology was evident in these women after complete investigation, and their male partner had an identifiable cause of infertility. Interpretative examples are colour coded in Table 3.6A; the blue figures show the mean TAC values and associated 'P' value for the (MF vs Others) difference, for fluid from follicles containing oocytes from MF and Tubal factor patients, while the red values refer to data for the same aetiological comparison (MF vs Tubal factor) but in samples from follicles which did not yield an oocyte.

Focussing first on differences in follicular fluid TAC related to aetiology of infertility at common outcomes (Table 3.6A; 'MF vs Others' P values), it was noted that TAC was significantly higher in the 'unexplained' infertility set than in the MF set, whether an oocyte was recovered from the follicle or not. There was also a tendency

for follicular fluid TAC to be elevated in the tubal factor set (vs the MF set) when an oocyte was present in the source follicle (P = 0.06).

Aetiology	Outcome	N	Mean TAC (mmol/l)	Outcome P	MF *vs* Others P
MF	*With oocytes*	89	0.633 ± 0.130	0.84	
	No oocytes	33	0.627 ± 0.149		
Tubal	*With oocytes*	44	0.698 ± 0.166	**0.03**	0.06
	No oocytes	15	0.564 ± 0.272		0.78
Unexplained	*With oocytes*	71	0.717 ± 0.142	0.15	**0.01**
	No oocytes	33	0.757 ± 0.105		**0.01**
MF	*Fertilised*	63	0.633 ± 0.152	0.96	
	Not fertilised	26	0.634 ± 0.144		
Tubal	*Fertilised*	34	0.726 ± 0.158	**0.03**	**0.01**
	Not fertilised	10	0.600 ± 0.166		1.00
Unexplained	*Fertilised*	59	0.732 ± 0.139	**0.05**	**0.01**
	Not fertilised	12	0.644 ± 0.138		1.00
MF	*Viable*	56	0.627 ± 0.148	0.38	
	Non viable	7	0.681 ± 0.186		
Tubal	*Viable*	25	0.713 ± 0.169	0.41	**0.05**
	Non viable	9	0.764 ± 0.120		0.83
Unexplained	*Viable*	42	0.724 ± 0.148	0.51	**0.01**
	Non viable	17	0.751 ± 0.137		0.91

Table 3.6A: Influence of aetiology of infertility on follicular TAC and early IVF reproductive outcome.

Indeed, the tubal factor and 'unexplained' sets consistently exhibited higher follicular fluid TAC, by approximately 9–15%, than the MF set when subsequent reproductive outcome was positive (i.e. oocyte present; oocyte fertilisation-competent; embryo viable), but generally not when negative, the instance discussed above ('unexplained' vs MF, oocyte absent) being the exception to the rule.

When the effect of outcome on follicular fluid TAC was assessed within aetiological sets (Table 3.6A; 'Outcome' P values), it was observed that in the tubal factor set, TAC was depressed by approximately 20% in follicles in which an oocyte was absent, rather than present. In addition, in both the tubal factor and 'unexplained' sets, TAC was depressed (by 17% and 12%, respectively) in follicles which yielded oocytes which were subsequently fertilisation-incompetent, rather than competent.

When TAC loss over 72 hr was assessed, it was found that aetiology and outcome had no effect on this parameter. No significant differences were noted in follicular fluid TAC loss when the total data set was analysed after being sifted for aetiology of infertility and reproductive outcome (Table 3.6B).

		N	Mean TAC loss (mmol/l)	Outcome P	MF *vs* Others P
MF	*With oocytes*	89	0.133 ± 0.15	0.61	
	No oocytes	33	0.127 ± 0.13		
Tubal	*With oocytes*	44	0.135 ± 0.07	0.62	1.00
	No oocytes	15	0.123 ± 0.10		1.00
Unexplained	*With oocytes*	71	0.112 ± 0.07	0.36	1.00
	No oocytes	33	0.127 ± 0.08		1.00
MF	*Fertilised*	63	0.114 ± 0.10	0.24	
	Not fertilised	26	0.141 ± 0.08		
Tubal	*Fertilised*	34	0.142 ± 0.08	0.23	0.41
	Not fertilised	10	0.110 ± 0.06		0.90
Unexplained	*Fertilised*	59	0.111 ± 0.07	0.91	1.00
	Not fertilised	12	0.114 ± 0.09		1.00
MF	*Viable*	56	0.115 ± 0.10	0.85	
	Non viable	7	0.108 ± 0.08		
Tubal	*Viable*	25	0.148 ± 0.08	0.44	0.37
	Non viable	9	0.124 ± 0.07		1.00
Unexplained	*Viable*	42	0.117 ± 0.07	0.33	1.00
	Non viable	17	0.097 ± 0.09		1.00

Table 3.6B: Influence of aetiology of infertility and early IVF reproductive outcome on the follicular TAC loss over 72 h.

MF infertility was associated with higher proportion of viable embryos following IVF treatment than the other aetiology groups (Table 3.6C).

In summary, a background of high follicular fluid TAC was apparent in women with tubal factor and 'unexplained' infertility, compared to the MF control set, especially when reproductive outcomes were positive. These results suggest that an aetiology-related alteration of follicular fluid prooxidant-antioxidant balance may play a role in infertility, as discussed in Section 3.5.

		N	Pearson Chi-square value	P
MF	*With oocytes*	89	0.933	0.63
	No oocytes	33		
Tubal	*With oocytes*	44		
	No oocytes	15		
Unexplained	*With oocytes*	71		
	No oocytes	33		
MF	*Fertilised*	63	3.374	0.19
	Not fertilised	26		
Tubal	*Fertilised*	34		
	Not fertilised	10		
Unexplained	*Fertilised*	59		
	Not fertilised	12		
MF	*Viable*	56	6.461	**0.04**
	Non viable	7		
Tubal	*Viable*	25		
	Non viable	9		
Unexplained	*Viable*	42		
	Non viable	17		

Table 3.6C: Influence of aetiology of infertility on the frequency of early IVF reproductive outcomes

Subject-by-subject analysis based on categorising the 34 subjects as having 'good' or 'poor' reproductive outcomes (Tables 3.7A, B and C), as discussed above in Section

3.4.2.2, broadly concurred with observations noted in the follicle-by-follicle analysis. When the effects of aetiology on TAC were considered (Table 3.7A), a large aetiological effect was apparent in the 'unexplained' group, in whom follicular fluid TAC was elevated at all 'good' and 'poor' reproductive outcomes, relative to the MF controls.

Outcome		N	Mean TAC (mmol/l)	Outcome P	MF vs Others P
Oocyte recovery (poor = <72%; good = ≥72%)					
MF	<72%	6	0.613 ± 0.140	0.13	
	≥72%	7	0.653 ± 0.147		
Tubal	<72%	4	0.521 ± 0.191	**0.01**	**0.01**
	≥72%	3	0.769 ± 0.142		**0.01**
Unexplained	<72%	4	0.741 ± 0.119	0.54	**0.01**
	≥72%	8	0.724 ± 0.140		**0.01**
*Fertilisation (poor = <78%; good = ≥78%)**					
MF	<78%	5	0.602 ± 0.147	0.09	
	≥78%	7	0.650 ± 0.144		
Tubal	<78%	4	0.585 ± 0.192	**0.01**	0.63
	≥78%	3	0.756 ± 0.181		**0.01**
Unexplained	<78%	4	0.707 ± 0.119	0.13	**0.01**
	≥78%	8	0.746 ± 0.138		**0.01**
*Embryo viability (poor = <79%; good = ≥79%)**					
MF	<79%	3	0.639 ± 0.209	0.47	
	≥79%	7	0.613 ± 0.124		
Tubal	<79%	3	0.753 ± 0.173	**0.01**	**0.05**
	≥79%	4	0.598 ± 0.203		0.62
Unexplained	<79%	9	0.719 ± 0.145	0.20	**0.05**
	≥79%	3	0.754 ± 0.097		**0.01**

Table 3.7A: Aetiology of infertility, reproductive performance and follicular fluid TAC in 34 women undergoing IVF. (**Sample size decreased due to complete absence of oocytes or fertilisation in some women*).

In addition, women with tubal factor infertility who exhibited 'good' (≥72%) oocyte recovery and 'good' (≥78%) fertilisation rates exhibited greater mean individual follicular TAC than did MF controls at these outcomes. Follicular TAC was also greater in the tubal factor set than in MF controls when embryo viability rates were 'poor' but not when 'good'. In contrast, TF women exhibited lower TAC than did MF controls when oocyte recovery was 'poor'.

With regard to the effects of reproductive outcome on follicular TAC within each aetiological group, all reproductive outcomes had no significant effect on TAC in the 'unexplained' and MF groups. In the tubal factor set however, follicular TAC was 32% lower in those with poor oocyte recovery rate, 22.6% lower in those with poor fertilisation rate, and 26% higher in those with poor embryo viability rate, all relative to those with good outcomes.

Aetiology had little effect on follicular fluid TAC loss over 72 hr in each reproductive outcome group. An exception to this rule was that among women with unexplained infertility and 'poor' fertilisation and 'poor' embryo viability tended to exhibit less TAC loss over 72 h than did the relevant control MF groups (Table 3.7B). Outcome effects on TAC loss were also scarce, although in the MF group, 'poor' rather than 'good' embryo viability was associated with greater TAC loss.

Individual mean TAC was less variably distributed in the 'unexplained' group (coefficient of variation (CV) = 7.6%) than in the TF (CV = 22.2%) and male factor

(CV = 18.8%) groups (Table 3.7C). This may suggest that common factors impact on follicular TAC to a similar extent in women with unexplained infertility.

Outcome		N	Mean TAC (mmol/l)	Outcome P	MF vs Others P
Oocyte recovery (poor = <72%; good = ≥72%)					
MF	<72%	6	0.128 ± 0.104	0.70	
	≥72%	7	0.121 ± 0.080		
Tubal	<72%	4	0.142 ± 0.092	0.39	0.55
	≥72%	3	0.124 ± 0.070		0.84
Unexplained	<72%	4	0.120 ± 0.082	0.72	0.68
	≥72%	8	0.114 ± 0.074		0.68
*Fertilisation (poor = <78%; good = ≥78%)**					
MF	<78%	5	0.138 ± 0.105	0.14	
	≥78%	7	0.111 ± 0.086		
Tubal	<78%	4	0.124 ± 0.081	0.41	0.50
	≥78%	3	0.141 ± 0.079		0.13
Unexplained	<78%	4	0.106 ± 0.074	0.25	0.09
	≥78%	8	0.123 ± 0.078		0.42
*Embryo viability (poor = <79%; good = ≥79%)**					
MF	<79%	3	0.165 ± 0.140	**0.02**	
	≥79%	7	0.111 ± 0.080		
Tubal	<79%	3	0.134 ± 0.082	0.87	0.36
	≥79%	4	0.130 ± 0.080		0.24
Unexplained	<79%	9	0.116 ± 0.082	0.93	**0.05**
	≥79%	3	0.117 ± 0.063		0.68

Table 3.7B: Aetiology of infertility, reproductive performance and follicular fluid TAC loss in 34 women undergoing IVF.

Subject ID	Mean TAC (mmol/l)	Mean TAC Loss (mmol/l)
Male factor		
532	0.562 ± 0.089	0.111 ± 0.069
62	0.820 ± 0.096	0.207 ± 0.111
082	0.735 ± 0.125	0.138 ± 0.149
674	0.595 ± 0.225	0.241 ± 0.144
461	0.629	0.181
106	0.573 ± 0.067	0.046 ± 0.036
617	0.600 ± 0.171	0.134 ± 0.062
654	0.650 ± 0.149	0.065 ± 0.053
593	0.711 ± 0.046	0.121 ± 0.038
360	0.783 ± 0.154	0.156 ± 0.110
569	0.600 ± 0.067	0.121 ± 0.043
686	1.029	0.115
700	0.734	0.042
Mean	**0.694 ± 0.131**	**0.129 ± 0.059**
Tubal factor		
618	0.802 ± 0.117	0.131 ± 0.070
566	0.771 ± 0.213	0.164 ± 0.097
659	0.474 ± 0.203	0.174 ± 0.085
665	0.456 ± 0.124	0.134 ± 0.089
359	0.720 ± 0.142	0.097 ± 0.051
666	0.586 ± 0.261	0.152 ± 0.103
523	0.752 ± 0.155	0.117 ± 0.137
Mean	**0.652 ± 0.145**	**0.138 ± 0.027**
Unexplained		
664	0.658 ± 0.144	0.06 ± 0.062
740	0.713 ± 0.165	0.012 ± 0.001
434	0.769 ± 0.153	0.186 ± 0.046
82	0.839 ± 0.051	0.161 ± 0.065
616	0.665 ± 0.103	0.115 ± 0.095
414	0.682 ± 0.113	0.134 ± 0.054
762	0.767 ± 0.033	0.110 ± 0.063
708	0.764 ± 0.241	0.086 ± 0.026
482	0.739 ± 0.146	0.133 ± 0.107
145	0.789 ± 0.082	0.106 ± 0.065
696	0.733 ± 0.117	0.096 ± 0.104
715	0.681 ± 0.074	0.105 ± 0.056
Mean	**0.733 ± 0.055**	**0.109 ± 0.045**
Endometriosis		
570	0.621 ± 0.118	0.134 ± 0.065
Endometriosis + Tubal		
537	0.892 ± 0.158	0.115 ± 0.083

Table 3.7C: Mean TAC within each patient according to diagnostic groups

3.4.3.2 *Effects of Confounders – data presentation*

The total data set for the 303 follicular fluid samples was grouped for analysis on the basis of the presence or absence of each confounding factor in turn. The series of complex tables that follow (Tables 3.8A – 3.10B) have 2 columns of 'P' values. The Outcome 'P' values relate to the significance of the effect of reproductive outcome (i.e. oocyte+/-, fertilisation+/- or embryo viability+/-) on the mean TAC and TAC loss within each confounder set (e.g. smoking+ or smoking-; PCO+ or PCO-), i.e. constant confounder status. The 'P' values under '+/-PCO', '+/-Age effect' and '+/-Smoking', relate to the effect of the presence or absence of the confounder on follicular fluid TAC and TAC loss in the same reproductive outcome set (e.g. oocyte+, fertilisation-, etc).

3.4.3.3 *Influence of PCO morphology*

Only 8.6% of the follicular fluid samples were derived from women with PCO morphology (PCO+, n = 26 of 303). A comparison of the total data set divided on the basis of the presence/absence of PCO morphology, irrespective of reproductive outcome, suggested that PCO morphology was associated with little change in follicular fluid TAC (Table 3.8A).

Differences in follicular fluid TAC were however noted when data was analysed according to PCO morphology and reproductive outcome (Table 3.8A). Focussing first on differences related to the presence of PCO morphology at common outcomes (Table 3.8A; 'PCO+/-' P values), TAC in follicular fluid associated with fertilisation-incompetent oocytes, was 32% greater in women with PCO rather than normal ovarian morphology. In contrast, the TAC of follicular fluid associated with oocytes from which non-viable embryos were derived, was 22% less in women with PCO

rather than normal ovarian morphology. Follicular fluid TAC was however similar in women with and without PCO morphology, irrespective of whether the follicle contained an oocyte.

Considering the effect of outcome (Table 3.8A; Outcome 'P' values), women with normal ovarian morphology exhibited the same interactions between outcome and follicular fluid TAC as were seen for the total data set in Table 3.2. Specifically, the

	N	Mean TAC (mmol/l)	SD	+/-Outcome P	+/-PCO P
PCO+					
Total	26	0.683	0.16		0.91
PCO-					
Total	277	0.679	0.16		
PCO+					
With oocytes	17	0.686	0.17	0.89	0.87
No oocytes	9	0.677	0.13		0.98
PCO-					
With oocytes	201	0.680	0.16	0.98	
No oocytes	76	0.679	0.19		
PCO+					
Fertilised	11	0.617	0.17	**0.02**	0.10
Not fertilised	6	0.814	0.10		**0.01**
PCO-					
Fertilised	158	0.699	0.15	**0.01**	
Not fertilised	43	0.613	0.14		
PCO+					
Viable	7	0.630	0.20	0.75	0.39
Non viable	4	0.594	0.10		**0.03**
PCO-					
Viable	127	0.683	0.16	**0.02**	
Non viable	31	0.758	0.14		

Table 3.8A: Influence of PCO morphology and early IVF reproductive outcome on follicular TAC.

presence of an oocyte had no influence on TAC, whereas fertilisation incompetence was associated with low TAC, and embryo non-viability with high TAC. This was not surprising as the women with normal ovarian morphology comprised 92% of the total data set. The presence of an oocyte also had no influence on follicular fluid TAC in women with PCO morphology, however in these subjects, oocyte fertilisation incompetence, rather than competence, was associated with a 32% increase in source follicular fluid TAC, the opposite to the effect observed in women with normal ovarian morphology. Embryo non-viability had no influence on TAC in the source follicle in women with PCO morphology, as opposed to the increased follicular fluid TAC seen in women with normal ovarian morphology.

There were no differences between follicular fluid TAC loss in women with PCO and in those with normal ovarian morphology when reproductive outcome was not included (Table 3.8B). When reproductive outcome was considered, follicular fluid TAC loss was similar whether the follicle contained an oocyte or not, irrespective of the presence/absence of PCO morphology. Follicular fluid associated with fertilisation incompetent oocytes did however show substantially greater (1.68-fold; $P = 0.020$) TAC loss in women with PCO than in women with normal ovarian morphology. Conversely, follicular fluid associated with oocytes which produced non-viable embryos tended to show a lower TAC loss in women with PCO (only 36% of the level in women with normal ovarian morphology; $P = 0.068$). No PCO-related differences were noted in TAC loss when reproductive outcome was positive.

With regard to outcome effects (Table 3.8B), TAC loss was not associated with differences in reproductive outcome in women with normal ovarian morphology, as

was observed for the total data set in Table 3.3. In contrast, in women with PCO morphology, TAC loss was more than 2-fold greater in follicular fluid associated with oocytes which were fertilisation-incompetent, rather than competent.

	N	Mean TAC loss (mmol/l)	SD	+/-Outcome P	+/-PCO P
PCO+					
Total	26	0.120	0.09		0.83
PCO-					
Total	277	0.124	0.08		
PCO+					
With oocytes	17	0.124	0.09	0.75	0.82
No oocytes	9	0.112	0.09		0.46
PCO-					
With oocytes	201	0.119	0.08	0.17	
No oocytes	76	0.135	0.09		
PCO+					
Fertilised	11	0.084	0.07	**0.01**	0.18
Not fertilised	6	0.197	0.10		**0.02**
PCO-					
Fertilised	158	0.120	0.08	0.87	
Not fertilised	43	0.117	0.07		
PCO+					
Viable	7	0.109	0.07	0.10	0.72
Non viable	4	0.041	0.03		0.07
PCO-					
Viable	127	0.121	0.09	0.71	
Non viable	31	0.115	0.08		

Table 3.8B: Influence of PCO morphology and early IVF reproductive outcome on follicular TAC loss over 72 h

PCO morphology had no influence on the frequency of reproductive outcomes in general (Table 3.8C).

	N	Pearson Chi-square value	P
PCO+			
With oocytes	17	0.607	0.50
No oocytes	9		
PCO-			
With oocytes	201		
No oocytes	76		
PCO+			
Fertilised	11	1.738	0.23
Not fertilised	6		
PCO-			
Fertilised	158		
Not fertilised	43		
PCO+			
Viable	7	1.756	0.24
Non viable	4		
PCO-			
Viable	127		
Non viable	31		

Table 3.8C: Influence of PCO morphology on the frequency of early IVF reproductive outcomes.

3.4.3.4 *Influence of age*

The total follicular fluid sample data set was subdivided into 2 age groups; ≤37 years and >37 years for the purpose of this analysis. This cut-off age falls into the age range (36-38 years) when the chance of a live birth following IVF treatment declines by 25%, from 20% to 15 % (NICE Guideline-Fertility, 2004). Although the optimal female age range for IVF treatment is 23-39 years (NICE Guideline-Fertility, 2004), an older cut-off age in this study was avoided because only 5 women were ≥40 years. Correlation/regression analysis was also used to determine the influence of the ages of the women on early reproductive outcome.

The mean age of women in the complete data set was 33.7 ± 5.0 years. When the data was divided into women aged ≤37 years (n = 225) and women aged >37 years (n =

75), but not by reproductive outcome, mean follicular fluid TAC levels were similar in the two age groups (Table 3.9A).

	N	Mean TAC (mmol/l)	SD	+/-Outcome P	Age effect P
Age >37					
Total	75	0.671	0.17		0.62
Age ≤37					
Total	225	0.682	0.16		
Age >37					
With oocytes	46	0.666	0.15	0.76	0.52
No oocytes	29	0.679	0.21		0.99
Age ≤37					
With oocytes	169	0.683	0.16	0.86	
No oocytes	56	0.679	0.17		
Age >37					
Fertilised	37	0.691	0.15	**0.02**	0.99
Not fertilised	9	0.565	0.14		0.12
Age ≤37					
Fertilised	131	0.692	0.16	0.21	
Not fertilised	38	0.655	0.16		
Age >37					
Viable	29	0.673	0.14	0.15	0.78
Non viable	8	0.757	0.15		0.64
Age ≤37					
Viable	105	0.682	0.16	0.19	
Non viable	26	0.729	0.14		

Table 3.9A: Influence of age ≤37 years or >37 years on follicular TAC and early reproductive outcome.

When data was analysed specifically for age effects on TAC ('Age effect P'; Table 3.9A), age appeared to have no effect on follicular fluid TAC and its interaction with reproductive outcome. When reproductive outcome (+/-Outcome) was considered however, failure of fertilisation was associated with low follicular fluid TAC in the women aged >37 years, but not in those ≤37 years. The association within the total set between non-viable embryos with an elevated follicular fluid TAC, noted in Table

3.2, was still apparent in both age groups, but not significantly so. This was probably due to the decrease sample sizes (n = 8 and 26). Further analysis without reference to reproductive outcomes, revealed that women aged >37 years generally had higher follicular fluid TAC loss than the younger group of women (Table 3.9B).

Age (years)	N	Mean TAC loss (mmol/l)	SD	+/-Outcome P	Age effect P
Age >37					
Total	75	0.144	0.09		**0.02**
Age ≤37					
Total	225	0.118	0.08		
Age >37					
With oocytes	46	0.143	0.08	0.96	**0.04**
No oocytes	29	0.145	0.10		0.35
Age ≤37					
With oocytes	169	0.115	0.08	0.39	
No oocytes	56	0.126	0.09		
Age >37					
Fertilised	37	0.142	0.10	0.85	**0.05**
Not fertilised	9	0.149	0.07		0.47
Age ≤37					
Fertilised	131	0.111	0.08	0.25	
Not fertilised	38	0.128	0.08		
Age >37					
Viable	29	0.141	0.10	0.95	0.14
Non viable	8	0.144	0.10		0.13
Age ≤37					
Viable	105	0.114	0.08	0.34	
Non viable	26	0.098	0.06		

Table 3.9B: Influence of age ≤37 years or >37 years on follicular TAC loss over 72 h and early reproductive outcome

When oocytes were recovered, age >37 years was associated with increased TAC loss relative to that in women aged ≤37 years. Increased TAC loss was also observed in women aged >37 years, relative to those age ≤37 years, in fluid from follicles from which fertilisation-competent oocytes were obtained.

Regression analysis did not show any significant correlation between age and oocyte recovery ($r = -0.20$; $p = 0.25$), age and fertilisation rates ($r = 0.23$; $p = 0.20$), or age and embryo viability ($r = 0.28$; $p = 0.13$).

3.4.3.5 Effect of smoking habit

The influence of smoking on the relationship between follicular fluid TAC, TAC loss and early reproductive outcome measures are shown in Tables 3.10A-C.

	N	Mean TAC (mmol/l)	SD	+/- Outcome P	+/- Smoking P
Smoking+					
Total	22	0.785	0.18		**0.01**
Smoking-					
Total	278	0.671	0.16		
Smoking+					
With oocytes	17	0.771	0.17	0.52	**0.01**
No oocytes	5	0.831	0.21		**0.05**
Smoking-					
With oocytes	198	0.672	0.15	0.91	
No oocytes	80	0.669	0.17		
Smoking+					
Fertilised	14	0.788	0.14	0.40	**0.02**
Not fertilised	3	0.693	0.30		0.54
Smoking-					
Fertilised	154	0.683	0.15	0.06	
Not fertilised	44	0.634	0.15		
Smoking+					
Viable	9	0.790	0.15	0.97	**0.03**
Non viable	5	0.785	0.15		0.41
Smoking-					
Viable	125	0.672	0.16	0.09	
Non viable	29	0.727	0.15		

Table 3.10A: Influence of smoking on follicular TAC and reproductive outcome

Smokers have a greater follicular fluid TAC when the total sample sets were compared between smokers and non-smokers, without considering reproductive outcome (Table 3.10A). When reproductive outcome was considered, smokers had elevated follicular fluid TAC, relative to non-smokers, in association with the presence or absence of an oocyte in the source follicle, with fertilisation-competent oocytes, and with oocytes that produced viable embryos.

	N	Mean TAC loss (mmol/l)	SD	+/-Outcome P	+/-Smoking P
Smoking+					
Total	25	0.115	0.08		0.59
Smoking-					
Total	278	0.125	0.08		
Smoking+					
With oocytes	17	0.094	0.06	**0.01**	0.16
No oocytes	8	0.186	0.11		0.16
Smoking-					
With oocytes	198	0.123	0.08	0.63	
No oocytes	80	0.129	0.08		
Smoking+					
Fertilised	14	0.092	0.06	0.73	0.22
Not fertilised	3	0.105	0.10		0.51
Smoking-					
Fertilised	154	0.120	0.09	0.35	
Not fertilised	44	0.134	0.08		
Smoking+					
Viable	9	0.095	0.07	0.82	0.36
Non viable	5	0.086	0.04		0.48
Smoking-					
Viable	125	0.122	0.09	0.60	
Non viable	29	0.113	0.08		

Table 3.10B: Influence of smoking on TAC loss over 72 hr and early IVF reproductive outcome.

With regard to outcome effects, failure of fertilisation and embryo non-viability tended (P = 0.064 and 0.088, respectively) to be associated with low TAC and high TAC respectively, in follicular fluid of non-smokers. These results mirror those for the complete data set shown in Table 3.2. These effects were also seen in smokers for fertilisation, but not significantly so because of small numbers. Follicular fluid mean TAC loss was comparable between smokers and non-smokers when the total data set was analysed (Table 3.10B). When effects of smoking and reproductive outcome on TAC loss were considered, smoking did not influence TAC loss at any reproductive outcome. In smokers, non-recovery of an oocyte was associated with high TAC loss in the source follicle compared to follicles that contained an oocyte. Smoking had no influence on the frequency of early reproductive outcomes (Table 3.10C).

	N	Pearson Chi-square value	P
Smoking+			
With oocytes	17	0.367	0.63
No oocytes	5		
Smoking-			
With oocytes	198		
No oocytes	80		
Smoking+			
Fertilised	14	0.192	1.00
Not fertilised	3		
Smoking-			
Fertilised	154		
Not fertilised	44		
Smoking+			
Viable	9	2.266	0.16
Non viable	5		
Smoking-			
Viable	125		
Non viable	29		

Table 3.10C: Influence of smoking on the frequency of early IVF reproductive outcomes.

Analysis of the effect of PCO morphology and smoking on follicular fluid antioxidants and reproductive outcome could not be performed when means for individual subjects were considered, because the limited number of women precluded valid statistical analysis.

3.4.4 *Comparing fertile and sub-fertile women in natural cycles (Study B)*

Follicular fluid antioxidant activity was compared between fertile and sub-fertile non-stimulated women undergoing laparoscopy. This was to determine the differences, if any, in follicular fluid TAC and TAC loss between the 2 groups of women during natural menstrual cycles.

Of the 27 patients recruited, a mature Graafian follicle was seen in 14 patients from whom follicular fluid was aspirated during laparoscopy. Several of the other women had already ovulated as evidenced by the presence of a corpus luteum. Nine of the patients in whom a mature Graafian follicle was observed were sub-fertile (experimental subjects), while 5 were apparently normally fertile and were undergoing laparoscopic sterilisation (control). Of the sub-fertile women, 5 had tubal pathology while 4 had 'unexplained' infertility.

As expected, parity was substantially less in sub-fertile women. The mean age of these women was similar to the mean age of IVF patients in study A (Table 3.11). For all subjects, the mean follicular fluid TAC was 0.934 ± 0.264 mmol/l. The mean follicular fluid TAC among the sub-fertile women was similar to that in fertile women. The mean TAC in this study was however much greater than in follicular fluid of stimulated women undergoing IVF.

Subjects	N	Mean age (years)	Mean parity	TAC (mmol/l)	TAC loss (mmol/l)
Sub-fertile	9	32.9 ± 3.18	0.33 ± 0.7	0.898 ± 0.251	0.149 ± 0.110
Fertile	5	35.2 ± 7.19	2.6 ± 0.9	1.0 ± 0.303	0.245 ± 0.206
P		0.40	**0.01**	0.51	0.27

Table 3.11: Comparison of fertile and sub-fertile subjects: age, parity, TAC and TAC loss

Follicular fluid mean TAC loss for all subjects was 0.183 ± 0.151. TAC loss among the sub-fertile patients was not different (P = 0.27) from that among fertile women. The follicular fluid TAC loss in these women was high compared to that of women undergoing IVF. The number of subjects investigated in this study was too small and variance too large, to show a statistically significant difference.

3.5 Discussion

In this Chapter, follicular fluid TAC and TAC loss over 72 h, were assessed as indirect measures of free radical activity. These parameters were correlated with early reproductive outcome in infertile women undergoing IVF. Previous studies have generally determined baseline levels of specific antioxidants and/or antioxidant enzymes such as SOD, ascorbate or GPx, as indirect measures of ROS and oxygen free radical activity. As discussed in Section 2.2, estimates of specific antioxidants or enzymes may not truly reflect the capacity of all the antioxidants in the samples to withstand oxidative stress. The combination of TAC and antioxidant consumption in this thesis was therefore aimed at providing a more comprehensive evaluation of oxygen free radical/ROS activity in follicular fluid.

Evidence from published reports is inconsistent with regard to the role of free radicals and/or antioxidants in female reproduction. When the total data set, undivided into aetiological sets, was assessed for relationships between follicular fluid TAC and reproductive outcome (Table 3.2), TAC had no association with the presence or absence of an oocyte, presumably because the oocyte is only one, albeit large, cell surrounded by a million or more other follicular cells. Its influence on follicular fluid may therefore be minimal. It is however possible that the oocyte may influence synthesis of components of ROS or TAC within other follicular cells via paracrine mechanisms and also that the follicular environment may affect the oocyte. Factors responsible for non-recovery of oocytes from follicles are not completely understood, though some studies suggested that this might be related to oocyte immaturity secondary to low hCG levels, an underlying ovulatory disorder or premature oocyte atresia (Awonuga et al, 1998; Esposito and Patrizio, 2000). It is clear from the results of the present study that non-recovery of oocytes is not generally related to variations in the concentrations of follicular antioxidants or indirectly oxygen free radicals/ROS.

Low follicular fluid TAC was however associated with subsequent oocyte fertilisation incompetence, while high follicular fluid TAC was associated with later embryonic non-viability. A follicular fluid TAC of approximately 0.680 mmol/l seemed optimal for positive early reproductive outcome. This association is similar for both the follicle-by-follicle analysis and for analysis based on poor/good outcomes in the 34 subjects. A difference between analyses was however apparent with regard to oocyte recovery, as follicular TAC was unaffected by oocyte recovery in the follicle-by-follicle analysis, whereas there was a trend ($P = 0.06$) towards greater TAC in women with good ($\geq 72\%$) versus poor ($<72\%$) oocyte recovery in the

individual analysis. The two analyses may not be precisely comparable however because the follicle-by-follicle analysis was specific with respect to outcome. In contrast, subject-by-subject analysis, with setting by whether individual performance was greater or less than the total average, meant the poor and good outcome sets were 'diluted' with some positive or negative outcomes, respectively. Only the subject-by-subject analysis suggested that a greater follicular TAC tended to support oocyte recovery, but both analyses indicated that greater follicular TAC was permissive for successful fertilisation, but lower TAC (increased free radicals) encouraged better embryo viability. High TAC might have supported/promoted fertilisation competence in some otherwise dysfunctional oocytes, which could therefore not produce viable embryos.

These results suggest a requirement for a fine prooxidant-antioxidant balance for successful reproductive outcome, at least in the IVF setting. Furthermore, the association between elevated follicular fluid TAC and embryo non-viability suggests that oxygen free radicals may have an important role during the final stages of oocyte maturation and in early embryonic development. The elevated follicular fluid TAC, associated in this study with embryo non-viability, may perhaps excessively quench free radicals in the preovulatory follicle, a critical level of which may be required for optimal oocyte maturation and early embryonic development. The presence of weakly reactive free radicals, such as $O_2^{\bullet -}$, has been shown to render oocytes developmentally more competent during *in vitro* maturation (Blondin et al, 1997). Furthermore, defective oocytes and embryos are predominantly derived from severely hypoxic (<3% O_2) follicles (Van Blerkom et al, 1997), supporting the requirement for an optimal level of oxygen free radical for oocyte, and subsequent

embryo, well being. These studies also support the suggestion from the present study that oxygen free radicals/ROS might play different roles depending on their concentrations and oxidising power, and the stage of oocyte/embryo development. This proposal also agrees with Attaran et al (2000) who suggested that an optimally elevated concentration of follicular fluid ROS might be a potential marker for predicting success in IVF patients, though the authors did not suggest the optimal level. An improvement in the accuracy of prediction of the outcome of IVF treatment would be extremely useful, since at present the accuracy of prediction of successful pregnancy is very poor, even when macroscopically high-quality embryos have been transferred.

Reproductive outcome was generally not influenced by follicular fluid antioxidant consumption (i.e. TAC loss) in the present study. Contrary to these results, Paszkowski and Clarke (1996) reported an association between increased consumption of antioxidants (suggested to be due to increased free radical generation) in pre-implantation embryo culture medium (PECM) and poor embryo development *in vitro*. This study was however different from those of Blondin et al (1997) and Attaran et al (2000), and the present study, because antioxidant activity was studied in culture medium rather than follicular fluid, and the presence of an embryo during culture may have influenced antioxidant activity, free radical generation or both, making it impossible to establish a cause-effect relationship between the state of the embryos and antioxidant consumption.

The use of both follicular fluid TAC and antioxidant consumption (TAC loss) in this thesis aimed to provide a clearer picture of the relationship between antioxidant activity and reproductive outcome. Follicular antioxidant consumption, though not a

true measure of free radical flux under physiological conditions, does provide an indicator of the potential of the fluid to counteract the effects of endogenously generated ROS or its capacity to withstand oxidative stress. Differences between samples will, in part, be due to the pro-oxidant potential of the fluid itself, which means that samples that are more rapidly depleted of antioxidants are more likely to have higher ROS/oxygen radical activity. In the present study, follicular fluid TAC loss did not appear to influence reproductive outcome when the entire sample set was analysed. Interesting observations were however noted when the aetiology of infertility and confounding factors were considered.

When the total data set was divided on the basis of aetiology of infertility and then analysed for relationships between TAC and outcome, it was of note that the significant differences observed for the total data set in Table 3.2, tended to be seen in the aetiological sets, albeit sometimes those differences were not significant. Occasionally however, the differences were absent or a contrary change occurred. Differences from the behaviour of the total set, and between the aetiological sets, may highlight the differing influence of free radicals and reactive oxygen species in specific aetiological conditions leading to infertility.

The tubal factor and 'unexplained' sets generally exhibited significantly higher follicular fluid TAC than did the male factor (MF) control group, when subsequent reproductive outcome was positive. This may suggest that oxygen free radical and ROS levels are elevated in women with 'unexplained' infertility or infertility related to tubal pathology, relative to (probably) 'normal' women whose male partners are sub-fertile. Thus positive early reproductive outcome is promoted in the 'unexplained' and tubal factor sets only if compensatory increases in follicular fluid

antioxidant levels are seen, at least in the IVF setting. The results in this thesis indicate that, for positive reproductive outcome, an optimal follicular fluid TAC exists for each aetiological condition causing infertility, due to different levels of free radicals and ROS related to that condition. In the follicle-by-follicle analysis of the total set this appeared to be approximately 0.680 mmol/l, in the tubal factor and 'unexplained' sets approximately 0.720 mmol/l, and in the MF 'control' set approximately 0.630 mmol/l. Therefore, a TAC value of 0.680 mmol/l for instance may have been too high in the MF group, and hence was associated with non-viable embryos.

Statistical comparison between the aetiological sets and the total data set was not performed since comparison between the aetiological subgroups was considered more valid, as the effect of aetiology on follicular fluid TAC and outcome needed to be assessed. In addition, the three aetiological sets analysed comprise 94% of the total data set, such that the results for the total set were effectively an aggregate of the results for the 3 aetiological sets. Certainly these results supports the hypothesis that follicular fluid TAC and oxygen free radical/ROS levels differ dependent on aetiology of infertility, and thus may have an important role in human reproduction.

In the total data set, there was no relationship between follicular fluid TAC and the presence of an oocyte. This was also the case for the 'unexplained' set and the MF control set in both the follicle-by-follicle and subject-by subject analyses. However in the tubal factor set, follicular fluid TAC was depressed when an oocyte was absent. This may again reflect the differing impact of free radicals and ROS on outcome dependent on the aetiology of infertility.

When an oocyte was recovered, subsequent fertilisation incompetence was associated with low follicular fluid TAC in the total data set, as well as in the tubal factor and 'unexplained' aetiological sets. In contrast, follicular fluid TAC was unrelated to subsequent oocyte fertilisation competence in the MF control set. With regards to embryo non-viability, this was associated with elevated follicular fluid TAC in the total data set. TAC was elevated to a similar degree in association with non-viable embryos in the tubal factor, MF control and unexplained sets, albeit the differences were not significant. The effect sizes remained similar however, so the loss of significance was probably because of the decrease in sample size, due to the separation of the total set into aetiological groups, and the low numbers reaching the final outcome.

The MF set was selected as a control group, since after clinical investigation to the same degree as the other subject couples, the female partner appeared 'normal' whereas the male partner had an identifiable cause of infertility. The 'unexplained' set was potentially contaminated with some 'normal' women whose male partners had undetected infertility, but this was unlikely since their semen had been found to be normal using standard classification. This contamination was therefore likely to be at a low level (if any) since few cases of male infertility remain undetected, and it is more likely that infertility is associated with the female, rather than the male, partner.

The apparently normal MF controls exhibited lower follicular fluid TAC than the other aetiological sets, and TAC was not correlated with reproductive outcome in the MF group. It is possible therefore that TAC levels in these subjects represent 'normal' levels, albeit during IVF, and that they exhibit little pro-oxidant stress. Also of interest, the greater proportion of viable embryos following successful fertilisation

found in women with male factor infertility (Table 3.6C) was most probably a reflection of the optimal condition of oocytes obtained from their ovarian follicles, further lending support to the consideration of this group as 'control'.

In the total data set, fertilisation-incompetence and embryo non-viability were associated with depressed TAC and elevated TAC levels, respectively, relative to the opposite outcomes. In contrast, in women with PCO morphology, fertilisation-incompetence and embryo non-viability were associated with elevated TAC and depressed TAC, respectively. This is very interesting, since it suggests a significant modification of the influence of free radicals/ROS and antioxidants, on reproductive outcome, with PCO morphology. The mechanisms involved in this modification are not clear and require further investigation. In non-PCO women, the reproductive outcome effects on follicular fluid TAC parallel those of the total data set, whereas an opposite effect again occurred with PCO morphology since high TAC was associated with fertilisation-incompetence compared with fertilisation-competence. Furthermore, follicular fluid associated with fertilisation incompetent oocytes also showed greater (1.68-fold; $P = 0.020$) TAC loss in women with PCO than in women with normal ovarian morphology.

In the presence of PCO morphology therefore, the fertilisation incompetent oocytes were retrieved from follicular fluid with greater TAC turnover (due to increased free radical generation), and a compensatory high TAC. As previously noted, such high follicular fluid TAC as was associated with fertilisation-incompetent oocyte in the PCO group, might be expected to excessively quench free radicals, causing a disturbance in follicular fluid prooxidant-antioxidant balance. On the other hand, follicular fluid from women with PCO morphology, associated with oocytes from

which non-viable embryos were derived, had low TAC and a tendency to a low TAC loss (only 36% of the level in women with normal ovarian morphology; P = 0.068). Such an environment may indicate excessive free radical activity. The mechanisms for these modifications of follicular fluid prooxidant-antioxidant balance, in the presence of PCO morphology, are not clear but may be related to any of the associated conditions including, increased androgen index, hyper-insulinism, tendency to ovarian hyperstimulation and/or anovulation.

The alterations of prooxidant-antioxidant balance probably operate at a later stage of female reproduction, than the time of embryo transfer considered in the present study, since PCO morphology had no significant influence on the frequency of early IVF outcomes (Table 3.8C). The implications of these data are not clear; especially since the presence of PCO morphology alone does not fulfil the criteria for the diagnosis of PCOS (Rotterdam PCOS consensus workshop, 2004).

With respect to age, the results in this thesis did not indicate an age influence on follicular fluid TAC as related to reproductive outcome. Women aged >37 years however had higher TAC loss than the younger group when the total data was assessed (Tables 3.9B), without considering reproductive outcome. The effect of age on TAC loss was also apparent when reproductive outcome was considered in the data analysis. In women aged >37 years, greater follicular fluid TAC loss was associated with oocyte recovery from the source follicle and with subsequent fertilisation-competence. This appears to be in conflict with Carbone et al (2003), who found that reproductive ageing was accompanied by a change in the antioxidant enzymatic pattern that could impair ROS scavenging efficiency in the follicular environment. In the present study, the increased follicular fluid TAC loss was

associated with a positive, rather than an adverse reproductive outcome. It does not appear therefore that an alteration of prooxidant-antioxidant balance could explain the widely known decline in female reproductive performance with advancing age (Klein and Sauer, 2001; NICE Guideline-Fertility, 2004). The outcome measures in the present study are however more specific to IVF and early events in oocyte/embryo development, whereas most studies on the subject of age and reproduction consider pregnancy rates as outcome measure, making comparison between studies difficult.

The effect of confounding factors on follicular fluid antioxidant activity was again apparent when smoking was considered. Follicular fluid from smokers had elevated TAC compared to fluid from non-smokers, and these elevated levels were apparent in association with all positive outcomes (i.e. presence of an oocyte, fertilisation-competent and embryo viability), as well as with absence of an oocyte. This was unexpected because cigarette smoke has been reported to induce high serum levels of free radicals, which have been generally implicated in smoking related diseases (Borish and Pryor, 1987). Paradoxical results have been reported with plasma TAC estimation under conditions associated with oxidative stress, such as kidney failure, metabolic disorders, and after strenuous physical exercise (MacKinnon et al, 1999; Sahlin et al, 1991). As plasma uric acid increases in these conditions, plasma TAC should increase because uric acid contributes significantly to plasma TAC. A similar consideration may explain the elevated follicular fluid TAC in smokers. Alho and Leinonen (1999) however reported no difference in plasma antioxidant activity between smokers and non-smokers. The present studies suggest that smoking actually reduces follicular fluid oxidative stress, contradicting Paszkowski et al (2002) who reported that active smoking induced intrafollicular oxidative stress. In smokers, both

viable and non-viable embryos were similarly associated with elevated TAC, in contrast to the pattern that emerged with the total set, and when aetiology was considered, where elevated TAC was associated with embryo non-viability. The lack of an association between smoking and poor early reproductive outcome contradicts studies by Baird and Wilcox (1985), Phipps et al (1987) and Hull et al (2000). The effects a high follicular fluid TAC in smokers might manifest later during embryonic/foetal development such that these effects were not apparent in the present study. A similar delay in the manifestation of the effects of prooxidant-antioxidant imbalance, or other insults, on the preovulatory oocyte might also provide an explanation for the low pregnancy rates following IVF treatment, despite the transfer of macroscopically normal embryos.

It was not possible in this thesis to ascribe a pregnancy to a particular follicle or embryo, since all subjects had at least two embryos transferred. The more valid final outcome measures therefore had to be early reproductive events before embryo transfer, in order to provide a clearer picture of how follicular fluid antioxidant status might be related to these measures. This made it difficult to directly compare results with those of other studies, such as those of Attaran et al (2000) where levels of ROS and TAC were compared between women who got pregnant and those who did not, following transfer of multiple embryos. These authors pooled follicular fluid samples from individual women, for their comparison. A similar attempt (mathematical pooling by finding subject mean TAC) in the present study to relate antioxidant activity to pregnancy showed a trend towards higher follicular TAC in women who did not get pregnant (0.724 ± 0.130 vs 0.634 ± 0.100 mmol/l; $p < 0.1$), agreeing with the association between high TAC and embryo non-viability. It should be emphasised however that this was essentially a crude analysis because it was impossible to

identify the embryo which resulted in a pregnancy and its associated follicular fluid, as normally two embryos were transferred into the uterus. It should be noted also that some women could have become pregnant from an oocyte obtained from follicular fluid excluded from the study because of blood/media contamination.

This present study was the first to evaluate antioxidant activity in follicular fluid from women during normal non-stimulated menstrual cycles. The mean follicular TAC and TAC loss over 72 h were not significantly different between sub-fertile subjects and the fertile controls, albeit the number of subjects studied was limited. The results however indicate a higher mean TAC in these women compared to the IVF patients, suggesting that the hormonal stimulation might reduce follicular antioxidants. It is also possible that the higher levels in natural cycles, compared to stimulated cycles is because, unlike in stimulated cycles, only one dominant follicle was usually present in the ovary during natural cycles. It would appear that follicular fluid TAC and TAC loss is similar in fertile and infertile women, suggesting that follicular antioxidant status and ROS were unlikely to play a critical role in non-IVF female reproductive outcome. However, larger studies are indicated to determine the effect of other factors, including aetiology and confounding factors, and to allow valid statistical analysis.

It is not surprising that the role of antioxidant supplementation and the therapeutic role of exogenous antioxidants in the treatment of male and female subfertility remain unproven. In the IVF setting for instance, the addition of ascorbate to the cryopreservation solution was reported to reduce levels of H_2O_2 in embryos and to significantly enhance inner cell mass development in blastocysts (Lane et al, 2002). On the other hand, Tarin et al (1994) found no beneficial effect of ascorbate when

they randomly allocated and cultured oocytes, spermatozoa and embryos with or without ascorbate. Furthermore, vitamins C and E and selenium are often used to treat male factor infertility but antioxidant levels do not appear to be reduced in these men, and the effectiveness of the treatment therefore remains controversial (Kessopoulou et al, 1995; Tarin et al, 1994; Suleiman et al, 1996; Donnelly et al, 1999). The results of the present studies suggest that supplementation may potentially interfere with the optimal prooxidant-antioxidant balance, depending on the aetiology of infertility and the presence of other confounding factors, and adversely affect fertility in women.

Oxygen free radicals and ROS influence human female reproductive outcome, however the evidence available in the literature provides a confusing picture of the mechanisms for this influence. Discrepancies between results of different studies are related to methodological differences, different biological samples studied, antioxidants/antioxidant enzymes examined, or the final outcome measures as discussed in Section 1.5.3 of the introduction. The effect of oxygen free radicals/ROS in relation to female reproductive function is complex. Embryos may also have different sensitivities to oxygen free radicals at different stages of development (Morales et al, 1999).

The hypothesis that follicular fluid oxygen free radicals, ROS and antioxidants influence female reproductive outcome was supported by the results of this thesis. The results suggest that an optimal follicular fluid prooxidant-antioxidant balance exists for successful female reproductive performance, a balance that varies with the aetiology of infertility, confounding factors such as smoking, oocyte maturation, and embryonic/foetal developmental stages. Further studies will be required to clarify the

physiological and pathological role of these entities at different stages of the human female reproductive cycle, especially beyond the time of embryo transfer.

Presentations and Publications

Oyawoye OA. Abdel Gadir A. Garner A. Perrett C. Constantinovici N. Hardiman P. Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. *Hum Reprod.* 2003; 18(11): 2270 – 2274.

Oyawoye OA. Abdel Gadir A. Garner A. Perrett C. Hardiman P. Comparison of follicular fluid antioxidant capacities between fertile and subfertile women. Oral presentation at the British Andrology Society, the British Fertility Society and the Society for Reproduction and Fertility meeting: *Fertility 2003* in Aberdeen, 13-17 July 2003.

OA Oyawoye. A Abdel Gadir. A Garner. C Perrett. Hardiman P. Comparison of follicular fluid antioxidant capacities between fertile and subfertile women. *Human Fertility.* 2003; 4: 227 [Abstract O43].

Chapter 4

The effect of Follicular Fluid Protein Concentrations on TAC and Early Reproductive Outcomes

Outline

This chapter addressed Aim 4 of the thesis - 'To investigate the protein content of follicular fluid and its relationship to total antioxidant levels (TAC) and early reproductive outcome'. The FRAP assay of TAC does not measure antioxidant capacity associated with thiol groups, especially in proteins, yet protein associated antioxidant activity in follicular fluid may influence reproductive outcome. This effect would not have been assessed in Chapter 3. Therefore there was a need to determine whether antioxidant activity due to follicular protein (especially albumin) had an influence on reproductive outcome. Follicular fluid protein concentrations were therefore estimated, as they should be approximately proportional to antioxidant activity associated with their sulphydryl content. The relationship between follicular fluid protein concentrations, follicular fluid TAC and early reproductive outcome was explored, to complement the results of the studies presented in Chapter 3. Furthermore, a preliminary assessment of follicular fluid glutathione concentrations was performed.

4.1 Introduction

Follicular fluid is first seen as an accumulation of fluid between the layers of granulosa cells in growing follicles, which fills the antrum as follicles develop from the pre-antral to antral state. It accumulates therein as the dominant follicle develops under the influence of FSH and LH into the mature Graafian follicle (Figure 1.3; Section 1.1.3).

Human follicular fluid is mainly a transudate from plasma, with additional components secreted by the oocyte, the granulosa cells surrounding the antrum and the theca interna and externa. These tissue layers surrounding the antrum, in addition

to the intraovarian capillary endothelial cells and basement membrane, form the 'blood-follicle barrier' separating capillary blood from follicular fluid. This barrier may regulate the flow of proteins and small molecules from blood into follicular fluid, thus influencing its composition. The degree of regulation is unclear, however large proteins produced by granulosa cells, such as proteoglycans, remain confined within the follicular antrum, suggesting granulosa cells have a 'blood-follicle barrier' function, at least to very large molecules (McConnell et al, 2002). Furthermore, the contents of follicular fluid alter during follicular growth and development and the levels of different components in follicular fluid do not necessarily parallel their serum concentrations. These observations again suggest that solute flux into the follicle is regulated, albeit some proteins may be secreted by the granulosa or theca cells into follicular fluid.

Assay methods for determining levels of vitamins and co-enzymes have been refined, and many studies have compared follicular levels of these substances with plasma levels. For example, using high-performance liquid chromatography, Cassano et al, (1999) reported 10-fold greater concentrations of ascorbic acid in follicular fluid of water buffalo than in plasma. Paszkowski and Clarke (1999) also investigated ascorbic acid in human follicular fluid using a commercial kit based on 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), the reduced form of which can be estimated by spectrophotometry. The total reducing activity in a follicular fluid sample towards MTT was measured, before and after treatment of the samples with L-ascorbate oxidase, the difference providing a specific estimate of ascorbic acid concentration. They reported higher levels (follicular fluid:serum ratio of 1.68) of ascorbic acid in human follicular fluid compared to serum.

Total protein concentration has been estimated to be lower in follicular fluid than in serum (Edwards, 1974) and, using the Biuret and Lowry protein assay methods, Manarang-Pangan and Menge (1971) found follicular fluid total protein concentration to be 50% of that in human serum. Indeed, Shapira et al. (1996) reported the total follicular fluid protein concentration was 3.8 ± 0.4 mg/ml, although the assay method used was not described. This estimate is however similar to those reported by Manarang-Pangan and Menge (1971) and Johnson (1973). In contrast, Shalgi et al (1972) estimated that human follicular fluid total protein concentrations were similar to that of serum.

Table 4.1 Protein concentrations in human follicular fluid (Johnson, 1973)

Protein species	**Molecular weight**	**Serum (mg/ml)**	**Follicular fluid (mg/ml)**	**% Serum values**
Albumin	66 KD	44.0	25.6	58
IgG	150 KD	13.0	5.8	45
Transferrin	90 KD	3.2	1.5	41
IgA	150-300 KD	2.1	0.8	36
Haptoglobin	75 KD	1.6	0.7	41
IgM	750 KD	1.4	0.2	12

Using radial immunodiffusion, the concentrations of various proteins in human follicular fluid were specifically determined (Johnson, 1973) and are shown in Table 4.1. This data supports the conclusion that the permeability of follicles to plasma proteins depends on their relative molecular mass. The developmental state of the follicle is also important and as the follicle matures, it becomes more permeable to plasma proteins (Spitzer et al, 1996). Most plasma proteins are therefore present in

follicular fluid, although levels differ from those in plasma in a manner suggestive of partially regulated protein transport from blood into the follicle.

The thiol (or sulphydryl) group are chemicals containing sulphur and hydrogen as found in amino acid cysteine and other molecules. Two sulphydryls can join to produce a disulphide bond. This group, which is present in a reduced state within coenzyme A, glutathione and many proteins, contributes to the overall physiological redox balance. Sulphydryls are not quantified by the FRAP assay because their reduction potentials are generally below that of the Fe^{3+}/Fe^{2+} half-reaction. They may however contribute significantly to follicular fluid antioxidant activity because of the presence of the reduced sulphydryl group. For example, Wayner et al (1985) determined plasma antioxidants in 7 human subjects using the total peroxyl radical-trapping antioxidant capacity assay. They found that vitamin E, urate and ascorbic acid were the major contributors to antioxidant capacity, however 57 - 73% of antioxidant activity was unaccounted for and could be partly attributed to the groups of plasma proteins, coenzymes and glutathione.

Glutathione (GSH, γ-glutamylcysteinylglycine) is a primary non-protein sulfhydryl synthesized in most cells. This ubiquitous tripeptide is formed by the ATP dependent condensation of glutamic acid and cysteine, catalyzed by γ-glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH. In addition to donating an electron during the reduction of H_2O_2 to water, GSH functions as a co-substrate in the metabolism of xenobiotics catalysed by glutathione S-transferases.

As a complementary study to those in Chapter 3, and in order to fully evaluate the relationship between follicular fluid TAC, ROS and human female reproductive

outcome, it was necessary to estimate follicular fluid thiols so that their levels could also be related to TAC, total follicular fluid protein concentrations and early reproductive outcome. Total protein concentrations were assessed as a surrogate for the 'thiol' antioxidant capacity which is not measured by the FRAP assay. Follicular fluid total protein concentrations were correlated with early reproductive outcome, as a surrogate for follicular fluid thiols, since protein and thiol concentrations should be directly correlated. A subsidiary rationale for measuring total protein concentrations was to control for the probability of follicular fluid dilution by the culture medium, as discussed below.

4.2 Methods

4.2.1 *Assay of total free thiols in follicular fluid*

This assay is based on the method of Sedlack and Lindsay (1968) using Ellman's reagent (DTNB; 5,5'-dithio-bis-nitrobenzoic acid) modified by Tietze (1969). To a mixture of 850 µl of PBS and 100 µl of 6 mM DTNB in a 1 cm semimicro cuvette, 50 µl of appropriately diluted follicular fluid was added. The sample was incubated at 25°C for 15 min. The absorbance was measured at 412 nm against a reagent blank. This absorbance is a measure of total free thiol and was converted to thiol concentration using an extinction coefficient for 5 thio-nitrobenzoate of 14,150 $M^{-1}cm^{-1}$ (Riddles et al, 1979).

Eight of the follicular fluid samples were also subjected to the FRAP assay (as described in Section 2.2.4) to determine the correlation, if any, between the thiols and TAC.

4.2.2 *Estimation of follicular fluid total protein concentrations*

The Bradford protein assay (Bradford, 1976) relies on the binding of Coomassie Brilliant Blue G250 to protein. The quantity of protein is estimated from the amount of dye in the blue anionic protein (bound) form, by measuring the absorbance of the assay solution at 595 nm. This is the most widely used method for estimating total protein in biological samples because it is simple, quick, sensitive and reproducible (Bradford, 1976).

Reagents

Coomassie Brilliant Blue G250 (Bio-Rad Laboratories, Hemel Hempstead. UK)

95% Ethanol (10 ml)

Phosphoric acid 20 ml of 85% w/v

Bovine serum albumin (1 mg ml^{-1}) (BSA; Sigma-Aldrich, UK)

To prepare the Bradford reagent, 20mg of Coomassie Brilliant Blue G250 (0.01% w/v) was mixed with 10 ml ethanol (0.8 M), 20 ml 85% phosphoric acid (1.7 M) and made up to 200 ml with distilled water. Bovine serum albumin (BSA) was used to prepare a standard curve.

Follicular fluid samples were diluted 250 times into PBS prior to assay (i.e. 10 µl follicular fluid was diluted to 2.5 ml).

A standard curve was prepared by pipetting duplicate 50 µl of 0, 40, 80, 120, 160 and 200 µg/ml of BSA into test tubes, then mixing with 950 µl of Bradford reagent. 50 µl of distilled water was used as reagent blank.

50 µl of the follicular fluid was thoroughly mixed with 950 µl of the Bradford reagent and allowed to stand for 10 mins. Spectrophotometric measurements were then taken at 595 nm against the reagent blank.

FRAP (Section 2.2.4) and Bradford protein assays were carried out on 44 randomly selected samples of follicular fluid from the set of 303 samples used in Chapter 3. Follicular protein concentrations were correlated with follicular fluid TAC and early reproductive outcome measures.

4.3 Statistics

A scatter plot was performed using Microsoft Excel (1997), and correlation coefficient was calculated using the SPSS program (version 11). All values are reported as means ± standard deviation.

4.4 Results

4.4.1 *Free thiols in follicular fluid*

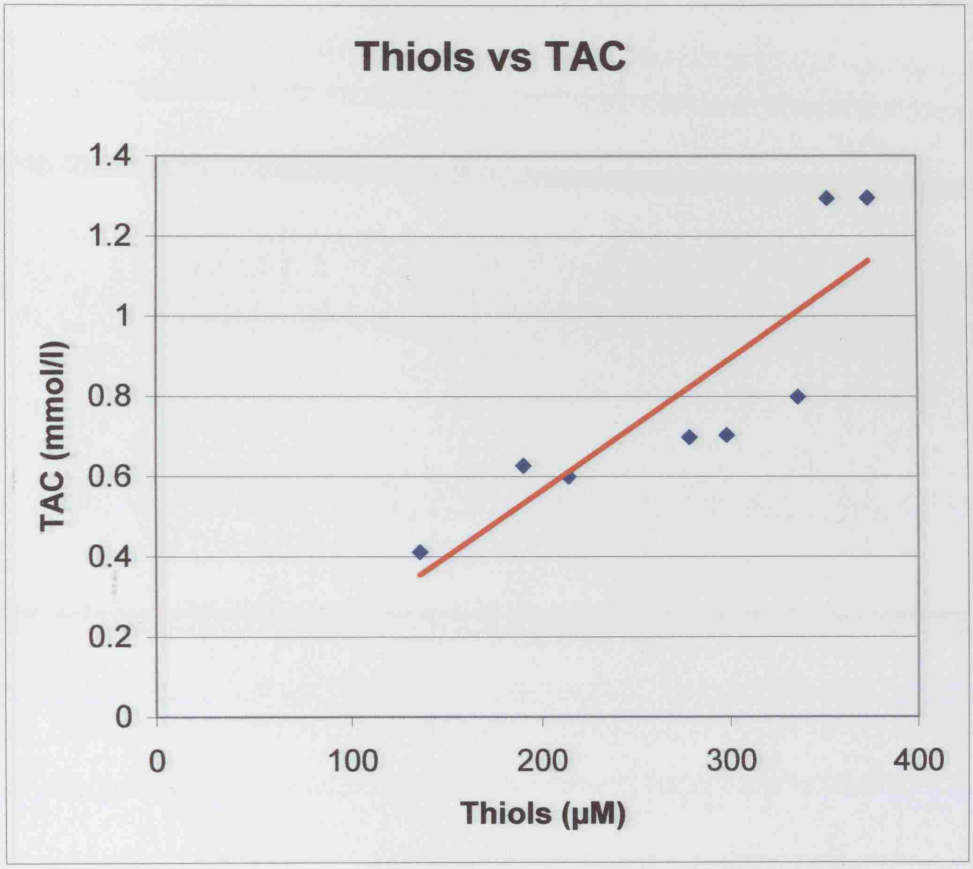
Eighteen randomly chosen human follicular fluid samples were assayed for free thiol content using the DTNB reagent. The mean value was 284 ± 82 µM, with upper and lower limits of 374 and 136 µM, respectively. This value amounts to about 40% of the magnitude of the FRAP estimated TAC value. The range of thiol values is therefore likely to substantially contribute to antioxidant activity in follicular fluid.

4.4.2 *Follicular fluid total protein concentrations*

The mean follicular total protein concentration was 37.4 ± 6.1 mg/ml. When follicular protein concentrations were compared with FRAP estimated TAC for the same sample (Figure 4.2), it was apparent no correlation existed between the two

parameters (r^2 = 0.001; P = 0.96), indicating that the variance in the FRAP estimates is not just a consequence of variations in protein content of the follicular fluid.

Figure 4.1 Relationship between follicular fluid thiols and FRAP estimated TAC



R^2 = 0.747; P = 0.006

There was also no correlation between follicular fluid total protein concentrations and early reproductive outcome. The tendency (P = 0.09) towards failure of fertilisation in association with low follicular fluid total protein concentration was probably a statistical artefact as a result of the small number of oocytes with this outcome (Table 4.2).

Figure 4.2: Follicular Protein Concentrations and Total Antioxidant Capacity

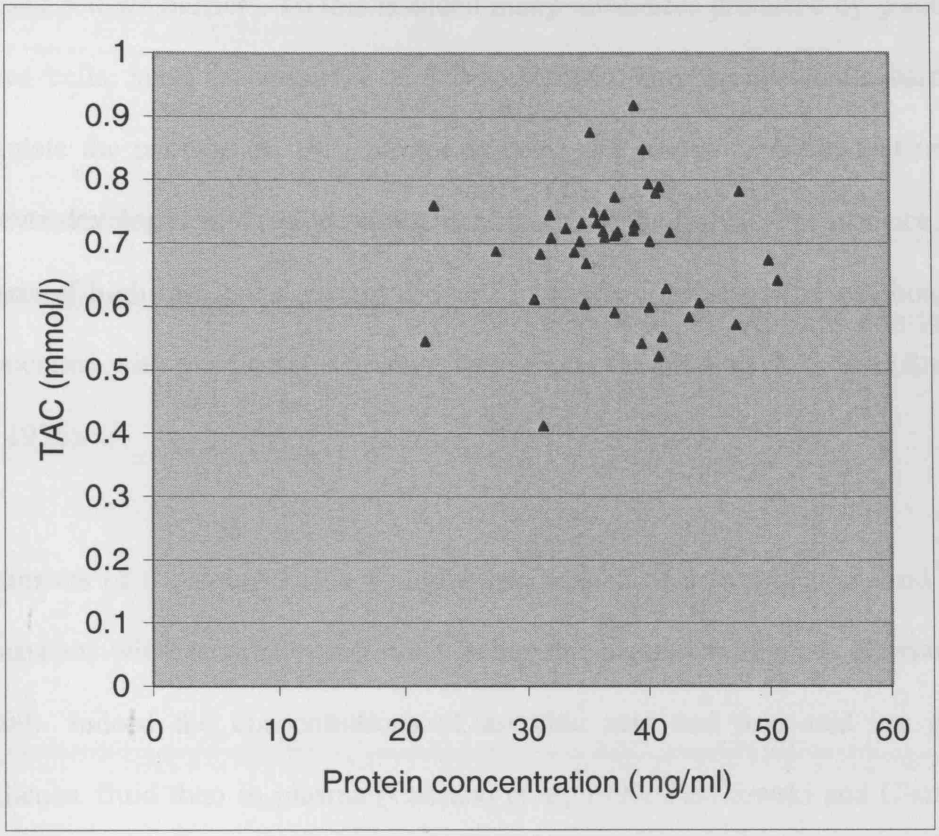


Table 4.2: Protein concentrations and early reproductive outcome

		N	Mean Total Protein mg/ml ± S.D	Standard Error of Mean	P
Oocyte retrieval	Yes	33	37.05 ± 5.53	0.96	0.53
	No	11	38.40 ± 7.65	2.31	
Fertilisation	Yes	31	37.46 ± 4.93	0.89	0.09
	No	2	30.67 ± 12.77	9.03	
Embryo viability	Yes	24	37.39 ± 4.86	0.99	0.88
	No	7	37.71 ± 5.55	2.10	

4.5 Discussion

Follicular fluid is, in part, a transudate of plasma components passing through the 'blood-follicle barrier'. To this is added many substances produced by granulosa and theca cells, such as oestradiol and progesterone. The 'blood-follicle barrier' may regulate the passage of cells, plasma proteins and other substances that may affect oocyte development, from perifollicular blood into the follicle. For instance, elevated levels of high molecular weight proteins in bovine follicular fluid are thought to be associated with poor oocyte quality, fertilisation failure, and/or atresia (Andersen et al, 1976).

Estimates of the contribution of individual antioxidants to follicular fluid TAC are consistent with ascorbate and urate being the major contributors (Cassano et al., 1999). Indeed the concentrations of ascorbic acid and uric acid are greater in follicular fluid than in plasma (Cassano et al, 1999; Paszkowski and Clarke, 1999) suggesting that uric acid and ascorbic acid are not freely diffusible from the follicle. The secretion of ascorbic acid might accompany the steroidogenic activity of granulosa cells, as has been found for hepatic cells (Nandi et al, 1997), or it may be actively transported from the blood into the follicle. Levels of ascorbic acid in cerebrospinal fluid (CSF) are also considerably higher than in plasma, though CSF TAC (estimated by TRAP assay), uric acid and sulphydryl concentrations are generally lower in CSF than in plasma (Alho and Leinonen, 1999).

Protein interference is a potential confounder in the measurement of total plasma antioxidant capacity (Wayner et al, 1985). Protein sulphydryl groups participate in the overall physiological redox balance and modulate oxidative stress, reversibly generating semi-oxidised species. Protein sulphydryl groups are responsible for an

element of the plasma TAC estimated by the TRAP and TEAC assays, because they react with peroxyl radicals during the assay reactions and may contribute to the duration of the lag phase in these assays (Ghiselli et al, 2000). The sulphydryl group in follicular fluid also contributes significantly (about 40%) to follicular antioxidants but this is not estimated by the FRAP assay, hence the necessity to determine the relationship between total protein concentrations, sulphydryls and early IVF reproductive outcome.

Interestingly, a good correlation exists between thiols (sulphydryls) and FRAP estimated TAC (Figure 4.1). Although sulphydryl levels were not directly correlated with reproductive outcome because of the limited number of samples, it follows that the influence of antioxidant activity due to these substances are likely to be similar to those described for TAC in Chapter 3, making the conclusions even more likely, having corrected for the main antioxidant group missed by the FRAP.

In the present study, the mean total protein concentration in follicular fluid of 37.4 $\pm$ 6.1 mg/ml was similar to values reported by Shapira et al. (1996), Manarang-Pangan and Menge (1971) and Johnson (1973). Carbone et al (2003) recently obtained samples by pooling fluids from 3 follicles per patient, and reported a mean protein concentration of 40.0 $\pm$ 2.0 mg/ml using the biuret method, which was comparable to our result. In contrast, Sabatini et al (1999) measured total protein in follicular fluid and serum by a colorimetric method using a Sigma diagnostic kit (Sigma 5412; Sigma Chemical Co., Poole, UK) and reported a concentration of 63.0 $\pm$ 0.69 mg/ml in follicular fluid, which was 16% less than in serum (76.66 $\pm$ 1.33 mg/ml). The levels were much higher than those obtained using other methods, including the Bradford assay in this thesis, presumably due to methodological differences. The

Sigma diagnostic kit may have been susceptible to interference with another follicular fluid component. Indeed the bicinchoninic acid, the protein-binding reagent used in this diagnostic kit, is known to interact with ascorbate and produce protein overestimation.

There is a significant amount of protein in follicular fluid, the concentrations of which did not influence early reproductive outcome. In studies where proteins from mature and immature human follicles were separated into different groups by gel electrophoresis (Spitzer et al, 1996) and immunochemical methods (Nagy et al, 1989), few differences were noted between the protein profiles from immature compared with mature follicles. Fluid from immature follicles contained fewer proteins and lower concentrations of proteins such as alpha-1-antitrypsin, suggesting that the variations in follicular fluid protein composition were mainly related to the follicular developmental stage (Spitzer et al, 1996; Nagy et al, 1989).

In the present study, there was no correlation between follicular fluid total protein concentrations and TAC. A close correlation was however not expected, since FRAP-estimated TAC does not assess antioxidant contribution of proteins, but might have suggested changes in variables that affect both parameters, such as accidental dilution of the fluid with flush buffer or contamination with blood. The total lack of correlation between these parameters also precludes the possibility that the variance in FRAP estimated TAC in this study resulted from decreased low molecular weight antioxidant levels as a consequence of accidental dilution of follicular fluid with flush buffer at the time of oocyte retrieval.

Chapter 5

Cytological Correlates of Follicular Fluid Antioxidant Activity

Outline

In this chapter, the relationship between the cellular components within follicular fluid from ovulatory follicles and the follicular fluid TAC is presented. This study tested the hypothesis that follicular fluid TAC may be influenced by different cell types within the fluid and impact on early reproductive outcome, and satisfied Aim 4, as detailed in Section 1.6.

5.1 Introduction

As discussed in Chapter 4, follicular fluid is an exudate of plasma, comprising water and solutes that pass from the capillary through the theca and granulosa cells comprising the follicular wall, to which is added the secreted products of cells found along this route. It also contains cells however, which may be present due to the sloughing off of granulosa cells from the lining of the antrum, as well as due to active cell migration into the fluid.

The cellular components of follicular secrete proteins and other substances some of which may influence folliculogenesis. Indeed, a major research objective regarding folliculogenesis is to identify these proteins and other substances within the follicular environment, as potential markers of follicular and/or oocyte dysfunction (Volpe et al, 1991). For example, using immunochemical techniques, Briggs et al (1999) showed that transferrin and its receptors are present in human granulosa cells, in which they are more pronounced in mature follicles. Transferrin binds Fe^{3+} and thus may reduce the generation of ROS and prevent follicular atresia. Follicular fluid transferrin concentrations are also correlated with follicular maturity and steroidogenesis, with the highest likelihood of oocyte fertilisation *in vitro* being associated with an intermediate transferrin concentration. This suggests that an

optimal degree of follicle maturation is required to maximise oocyte fertilisation competence (Entman et al, 1987).

Brannstrom et al (1994) determined the presence and localisation of leukocyte subsets in the human follicular wall and corpus luteum throughout the menstrual cycle, by immunohistochemical staining of ovarian tissue sections using a panel of leukocyte subtype specific monoclonal antibodies. Macrophages and neutrophilic granulocytes were found in high numbers in the follicular theca layer at ovulation. T-lymphocytes were present in the follicle wall and corpus luteum in low numbers, without significant variation in abundance throughout the menstrual cycle. The implications of these observations are unclear, though Brannstrom et al (1994) suggested that ovarian leukocytes might play a role in tissue remodelling and regulation of steroidogenesis during ovulation, luteinization and luteolysis. The location and timing of ovarian macrophage infiltration suggests that they may have a role in the phagocytosis of dying or damaged cells (Best et al, 1996).

Follicular fluid has also been shown to have chemotactic activity toward neutrophilic granulocytes and macrophages (Arici et al, 1996). Lei et al (1991), Brannstrom et al (1994), Best et al (1996) and Enien et al (1998) all isolated red blood cells, neutrophils, granulosa cells, lymphocytes, monocytes and macrophages from follicular fluid. Using the May-Grunwald-Giemsa stain and immunostaining with a mouse antibody against human leukocyte common antigen, Enien and his colleagues (1998) observed that leukocytes constituted 4 - 15% of the cellular component of follicular fluid. These studies suggested that follicular fluid contains a heterogeneous cell population, consisting of granulosa cells at different stages of differentiation, macrophages and lymphocytes (Lei et al, 1991; Brannstrom et al, 1996; Best et al,

1996). In follicular fluid and tissue sections of ovaries from 20 women undergoing IVF, Loukides et al (1990) found that granulosa-luteal cells predominate in follicular fluid but that resident macrophages and monocytes comprised 5 - 15% of cells. Interestingly, no correlation was observed between the number of monocytes or macrophages in the follicular fluid and the presence or absence of an oocyte in the follicle, or the cause of infertility.

Cells within follicular fluid, especially macrophages, secrete Interleukin-1β (IL-1β) which may influence pre-ovulatory follicular maturation. For example, addition of human recombinant IL-1β to ovaries of immature rats in an *in vitro* perfusion system resulted in a 3-fold increase in LH-induced ovulation rate (Brannstrom et al, 1993). This effect was thought to be partly mediated by increased production of progesterone. In contrast Mendoza et al (2002) found lower levels of follicular fluid IL-1β were associated with increased pregnancy rates during IVF. Assays were however performed on pooled follicular fluid samples from each patient because multiple embryos were transferred. It was impossible to say which oocyte led to pregnancy, thus specific association between the outcomes for an individual oocyte and the IL-1β level in the source follicular fluid could not be made.

Lachapelle and colleagues (1996) analysed leukocyte subpopulations in follicular fluid from infertile patients undergoing IVF, by flow cytometry using leukocyte sub-type specific monoclonal antibodies. Leukocytes from patients with idiopathic infertility had a higher proportion of T-lymphocytes than patients with tubal factor or endometriosis-related infertility. In contrast, patients with endometriosis had higher proportions of natural killer cells, B-lymphocytes, and monocytes. It was suggested

that these differences might in some way affect folliculogenesis and oocyte maturation.

Macrophages and neutrophils are potential sources of oxygen free radicals and ROS in follicular fluid. The studies described in Chapter 3 indicate the presence of free radical activity in the follicular environment, but the contribution of cells within the follicular fluid to the generation of these free radicals has not been fully explored. This study aimed to characterise and quantify the cell types within follicular fluid and relate them to follicular fluid TAC, as an indirect evaluation of their contribution to oxygen free radical activity.

5.2 Materials and Methods

To determine the cell concentration, follicular fluid was obtained from women undergoing IVF and embryo transfer. Ovulation induction, follicular tracking and follicular fluid/oocyte recovery were carried out as described in Section 2.3.2. Follicular fluid that was significantly contaminated with blood or culture medium was discarded. The volume of the fluid obtained was recorded and the oocyte was removed when present. The follicular fluid was then centrifuged at 600 g for 5 min and the supernatant aspirated and stored in fresh tubes at -80° C for subsequent determination of TAC by the FRAP assay, as described in Section 2.3.2.2. The cell pellet was left in 1 – 3 ml of follicular fluid with care taken to prevent any loss of cells. The cell suspension was stored at 4^{0}C for no longer than 12 h until processed for cell counting and staining. The volume of the cell suspension was first measured with a pipette at the time of cell count. The final total cell density estimates were corrected for the concentration factor related to the variability of the initial total follicular fluid volume and the suspension volume.

Cell count and staining were performed in the Department of Histopathology, Royal Free Hospital London. To perform cell counts, a 100µl aliquot of follicular fluid cell suspension was diluted as necessary in RPMI (Roswell Park Memorial Institute) medium to give a slightly turbid suspension. Cell counting was performed using a Neubauer counting chamber (Neubauer, Marienfeld, Germany) on a phase contrast microscope (x100 magnification), as recommended by the manufacturer. Cells in the 4 corner blocks (9 squares in each block) were counted. The cell density was calculated as:

Cells per mm^3 = Number of cells counted x 2.5 x Dilution factor in RPMI

Cell slides and staining were prepared for microscopy to identify the different types of cells present in follicular fluid. To prepare cells on glass slides for staining, approximately 50 µl of cell suspension were centrifuged onto a glass slide in a Cytospin centrifuge (Shandon Scientific Ltd., Astmoor, UK) at 950 rpm for 2 mins. Four slides were prepared for each cell suspension. Two were wet fixed for 20 mins in industrial methylated spirits (IMS) for Papanicolaou (Pap) staining, while two slides were air-dried and fixed for 10 minutes in 95% IMS for May-Grunwald-Giemsa (MGG) staining.

Pap staining was performed using an automated staining machine. Slides were rehydrated from 100% (v/v) IMS using 70% (v/v) IMS, 30%(v/v) IMS and distilled water, and were then stained with Harris' haematoxylin (Shandon International, Runcorn, UK) for 4 mins. After rinsing in tap water, the slides were differentiated in 0.5% (v/v) alcohol for 15 seconds and blued in tap water for 4 minutes. After dehydrating in 3 changes of IMS, slides were stained in Orange G (Merck Darmstadt,

Germany) for 1.5 mins, washed in 3 changes of IMS then stained in EA50 (Merck Darmstadt, Germany) for 1.5 mins. Slides were washed in 5 changes of IMS, cleared in 3 changes of xylene and mounted using neutral distrene-dibutylphthalate-xylene mounting medium (DPX; BDH Laboratory Supplies, Poole. UK).

To perform MGG staining, the fixed slides were placed in 50/50 May-Grunwald solution (Merck Darmstadt, Germany) for 15 mins then in 10% Giemsa buffer solution (Merck Darmstadt, Germany) for 20 mins. Slides were rinsed in MG buffer and left to stand in Sorenson's phosphate buffer, pH 6.8, for 5 minutes. They were then blotted dry, cleared in xylene and mounted in DPX.

5.3 Results

Twenty-two follicular fluid samples were processed for cytological assessment and FRAP assay, as described above and in Section 2.3.2.2.

The cellular component of follicular fluid consisted of granulosa cells, macrophages, lymphocytes, neutrophils and red blood cells. Cell types were clearly identified microscopically following staining (Figure 5.1a-c).

The average total cell concentration in follicular fluid was 1337 cells/mm^3. The mean proportion of inflammatory cells relative to the total cell density was 24 %. There was however wide variation in the density and proportion of granulosa and inflammatory cells between different follicles from the same patient and between patients (Table 5.1). The mean TAC and TAC loss were 0.686 $\pm$ 0.180 mmol/l and 0.116 $\pm$ 0.108 mmol/l, respectively.

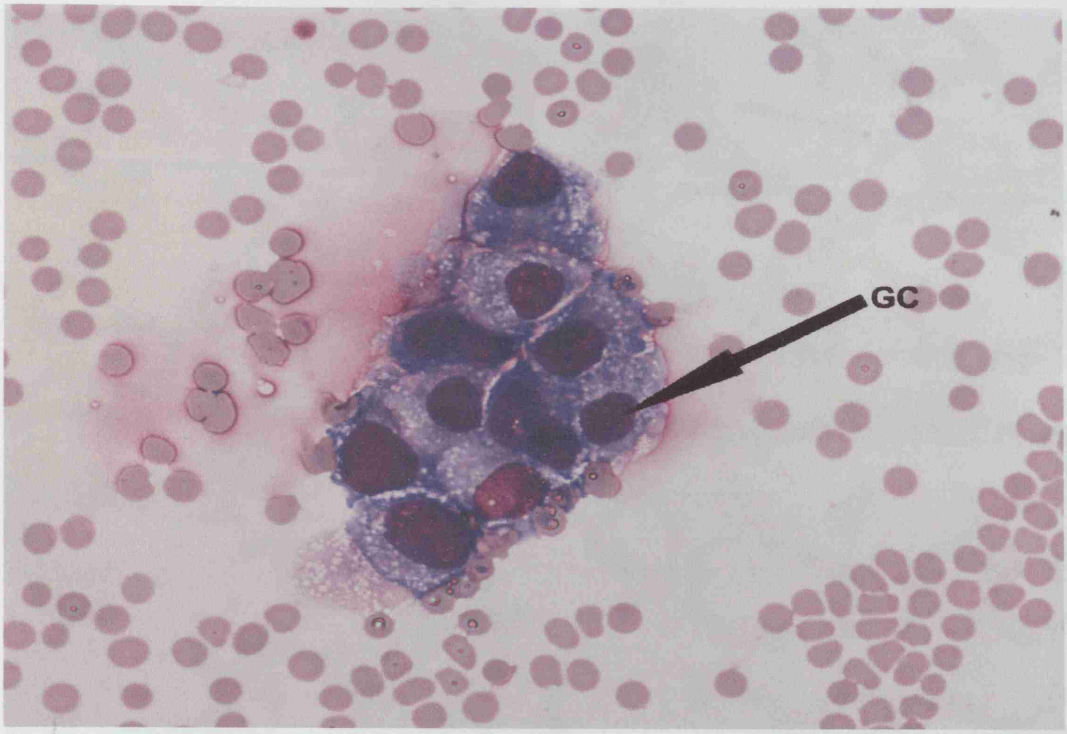


Figure 5.1a: Granulosa cells (GC); vacuolated cytoplasm more visible [MGG, x400]

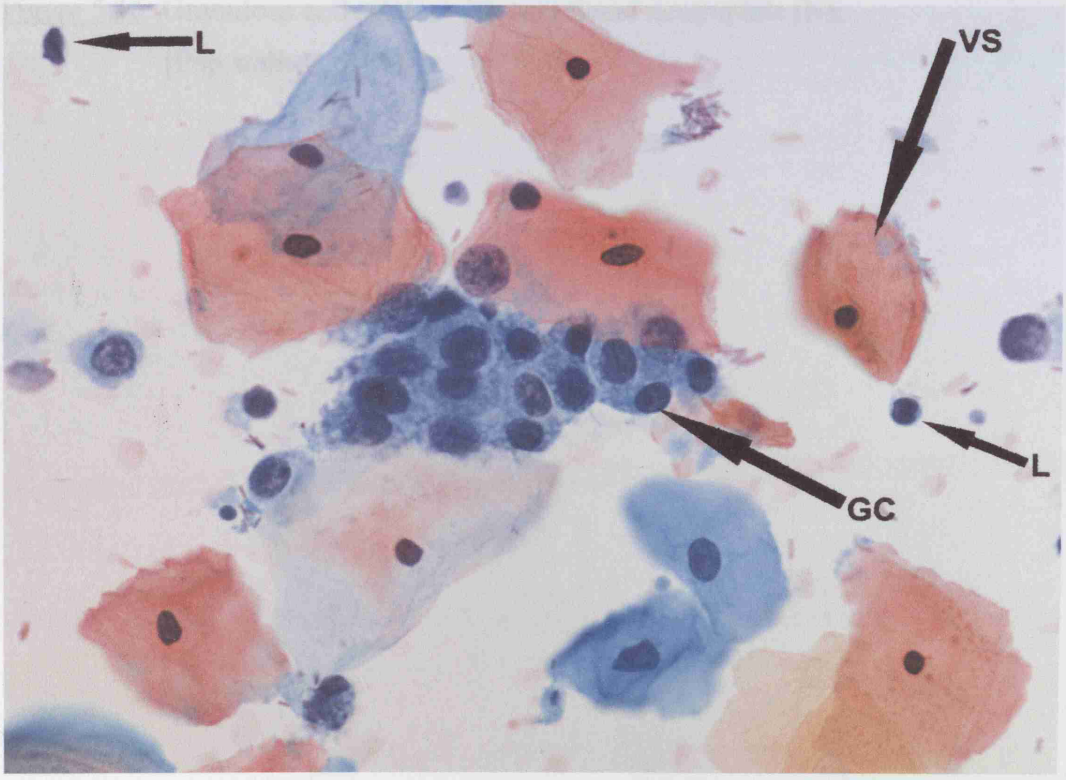


Figure 5.1b: Follicular cells (GC) with contaminant vaginal squames (VS), Lymphocytes (L); [Pap stained, x200]

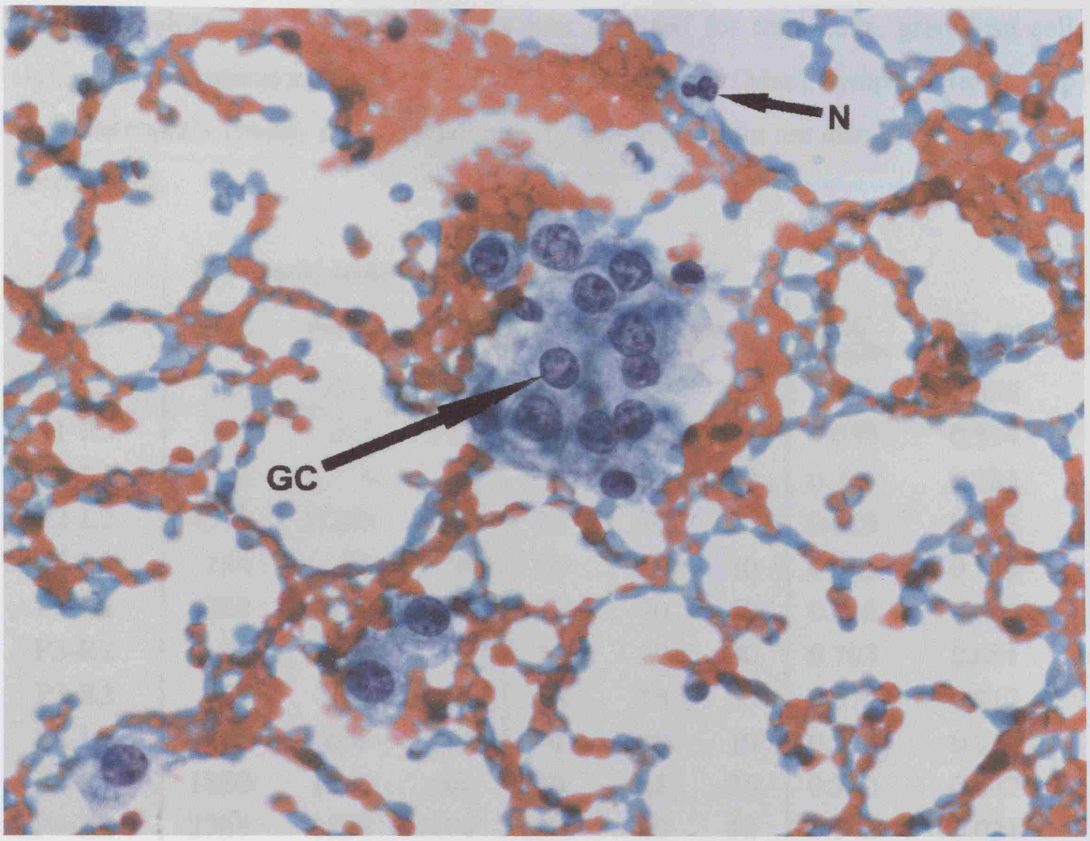


Figure 5.1c: Granulosa cells (GC) with occasional neutrophils (N);
[Pap stained, x400]

Table 5.1: Cell density, TAC and TAC loss over 72 hr in follicular fluid from patients undergoing IVF. Cell density was assessed for total cells, granulosa cells (GC) and inflammatory cells (IC). The % macrophages (Mac), lymphocytes (Lymp) and neutrophils (Neut) in the inflammatory cell population are also given. ND = not determined.

	Cell density (cells/mm^3)			Cell percentages				
Sample	**Total**	**GC**	**IC**	**Mac**	**Lymp**	**Neut**	**TAC (mmol/l)**	**TAC loss (mmol/l)**
P1-L3	686	446	240	30	50	20	0.918	0.358
P1-R3	315	205	110	30	45	25	1.030	0.334
P2-L1	5	4	1	ND	ND	ND	0.785	0.082
P2-L2	4571	3200	1371	20	30	50	0.629	0.003
P3-L2	144	86	58	60	10	30	0.825	0.319
P3-L4	264	158	106	30	20	50	0.516	0.069
P3-R2	45	27	18	50	20	30	0.703	0.081
P3-R3	227	182	45	50	15	35	0.574	0.017
P4-L1	820	656	164	0	60	10	0.518	0.016
P4-R1	1880	1316	564	30	45	25	0.710	0.077
P4-R2	2368	1894	474	30	60	10	0.542	0.024
P5-L1	394	355	39	50	30	20	0.959	0.094
P5-R1	3184	2544	637	30	60	10	0.699	0.142
P5-R2	11160	10044	1116	20	40	40	0.682	0.191
P6-R1	310	248	62	35	50	15	0.864	0.174
P6-R2	227	193	34	30	50	20	0.414	0.049
P6-L1	69	59	10	30	50	20	0.867	0.229
P7-L2	816	694	122	30	50	20	0.560	0.014
P7-L4	740	592	148	30	50	20	0.534	0.045
P7-L6	747	635	112	30	50	20	0.504	0.046
P7-L10	434	326	108	30	50	20	0.413	0.053
P7-R1	9	7	2	ND	ND	ND	0.837	0.130

The relationship between follicular cells and TAC or TAC loss is as depicted in Figures 5.2 to 5.7.

Figure 5.2: The relationship between granulosa cell density and follicular TAC

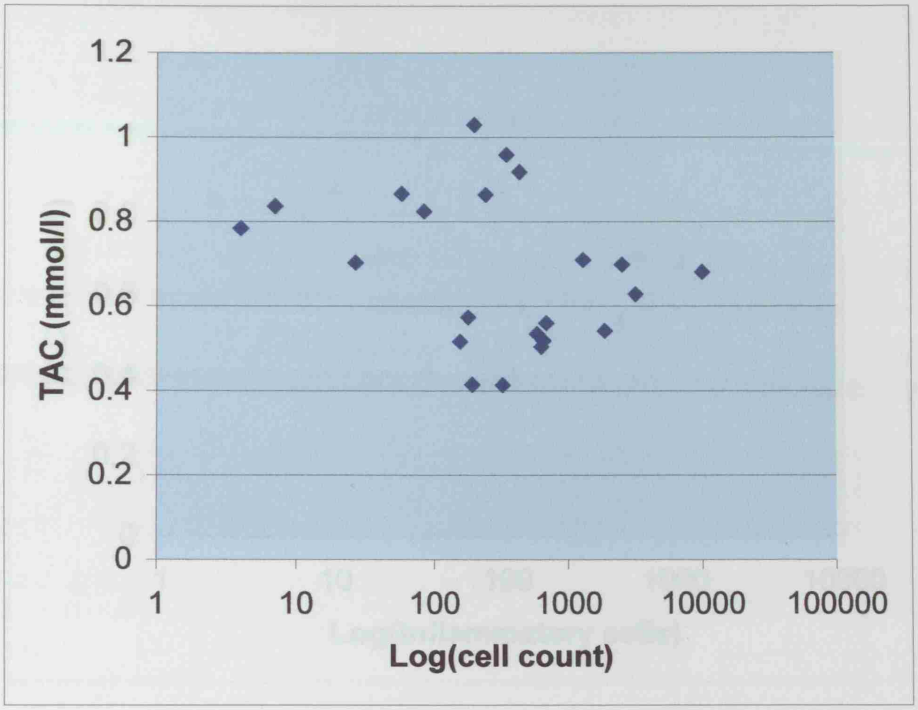


Figure 5.3: The relationship between granulosa cell density and follicular fluid TAC loss

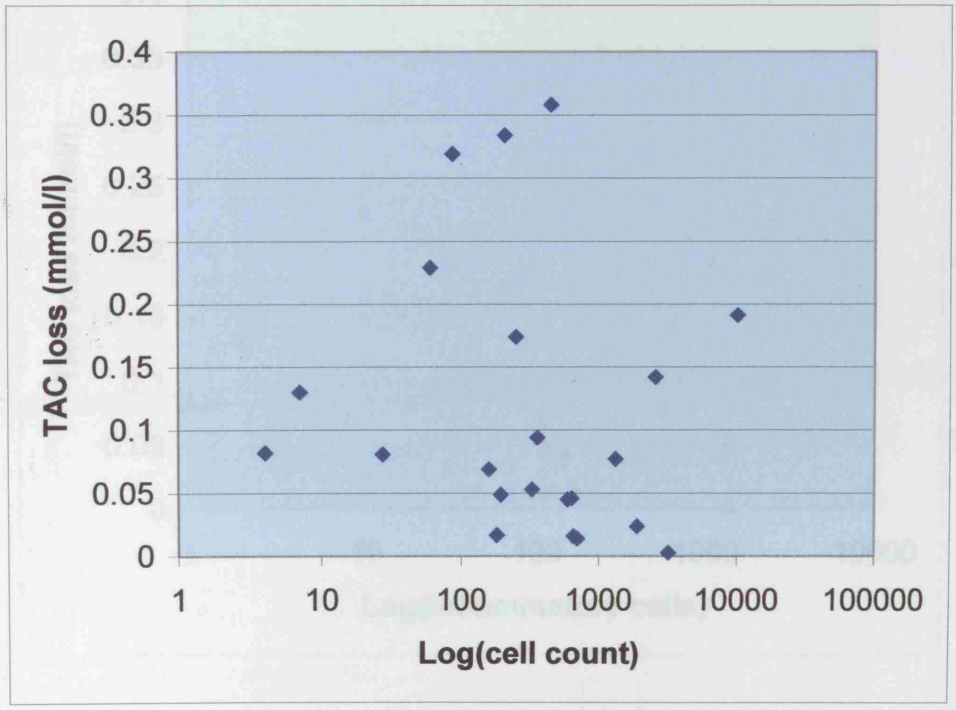


Figure 5.4: The relationship between inflammatory cells and follicular fluid TAC

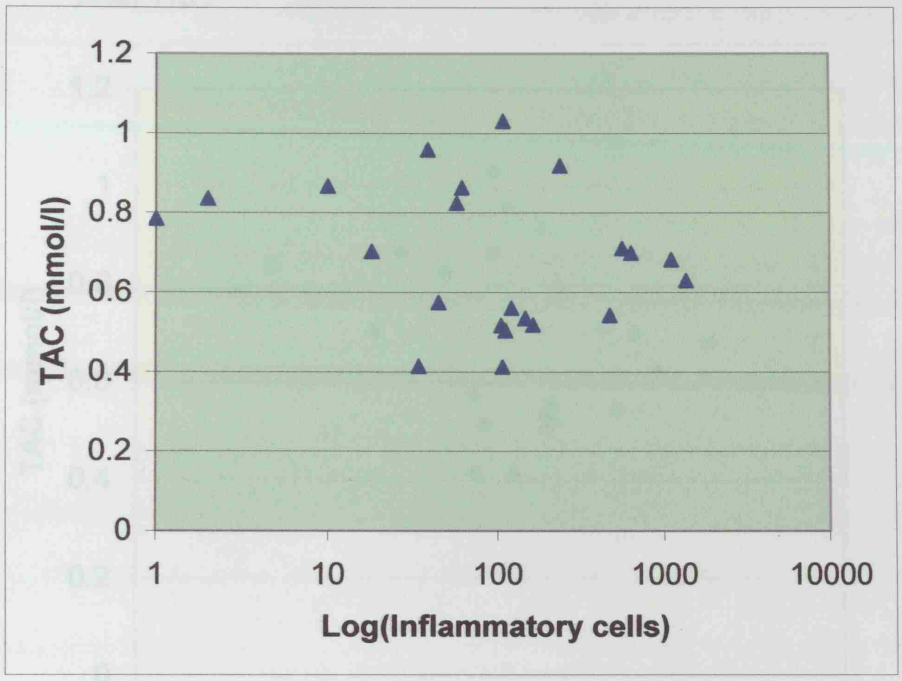


Figure 5.5: The relationship between inflammatory cells and follicular fluid TAC loss

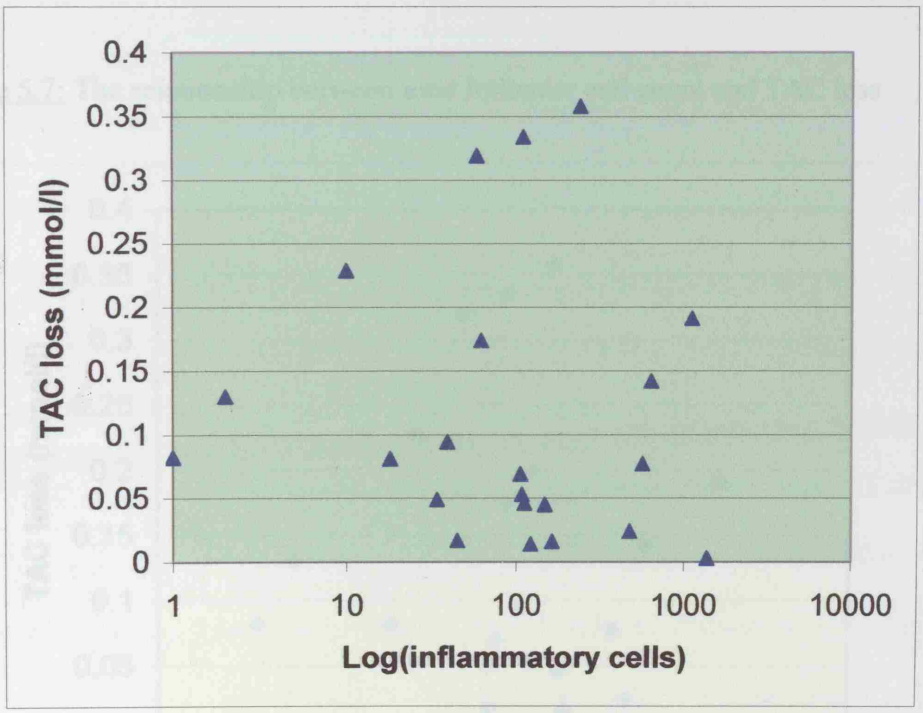


Figure 5.6: The relationship between total follicular cell count and follicular Fluid TAC

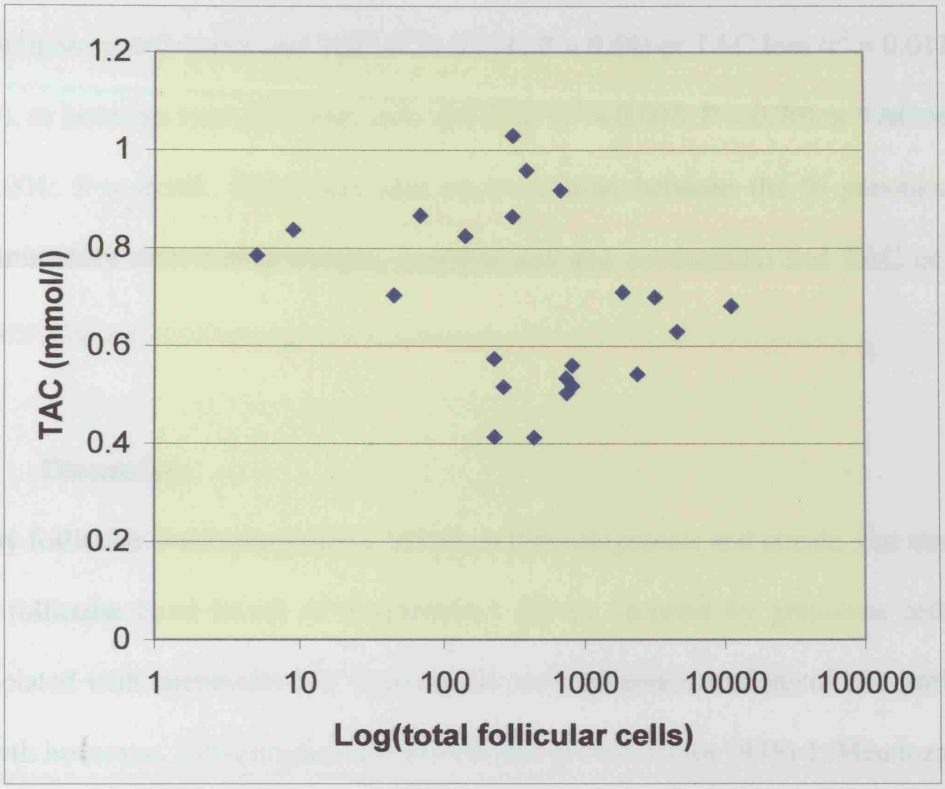
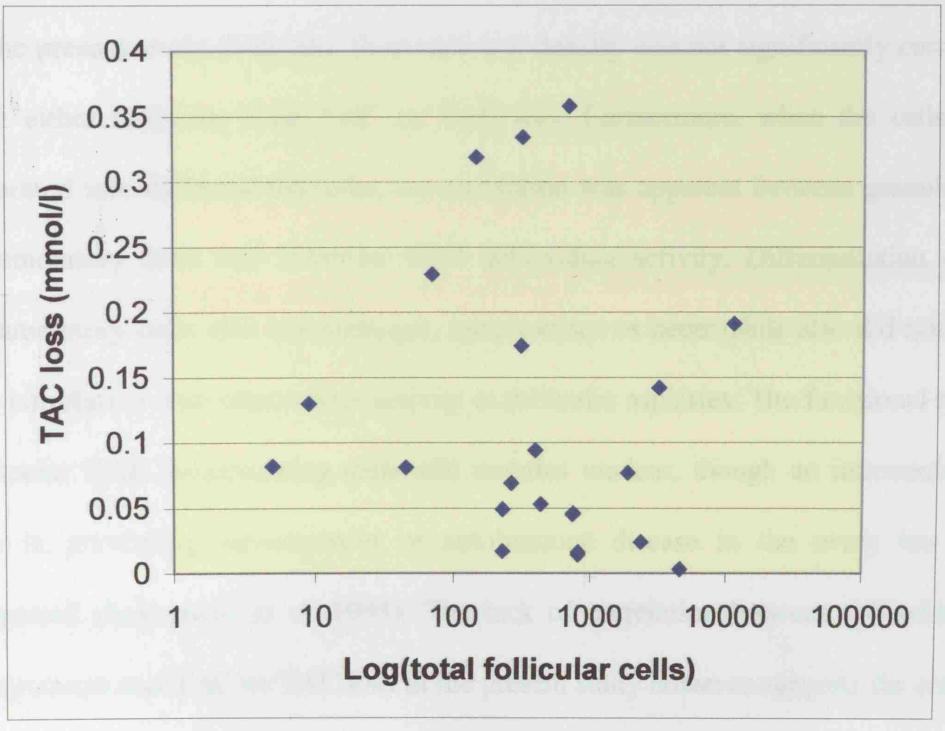


Figure 5.7: The relationship between total follicular cell count and TAC loss



There was no correlation between granulosa cell count and TAC (r^2 = 0.007; P = 0.72) or TAC loss (r^2 = 0.001; P = 0.90), nor was there a correlation between inflammatory cell count and TAC (r^2 = 0.011; P = 0.64) or TAC loss (r^2 = 0.011; P = 0.64), or between total follicular cells and TAC (r^2 = 0.008; P = 0.70) or TAC loss (r^2 = 0.001; P = 0.96). There was also no correlation between the % prevalence of inflammatory cells (macrophages, lymphocytes and neutrophils) and TAC or TAC loss.

5.4 Discussion

Many follicular fluid components influence folliculogenesis and atresia. For instance, low follicular fluid levels of Interleukin-1 (IL-1), secreted by granulosa cells, are associated with successful IVF outcome, as are high concentrations of LH, prolactin, growth hormone, 17ß-estradiol and insulin-like growth factor (IGF)-I (Mendoza et al, 2002). The relationship between these substances, oxygen free radical activity, antioxidant activity, and follicular fluid cell types has not been clearly defined.

In the present study, follicular fluid total cell density was not significantly correlated with either follicular fluid TAC or TAC loss. Furthermore, when the cells were separated into different fractions, no correlation was apparent between granulosa or inflammatory cells and follicular fluid antioxidant activity. Differentiation of the inflammatory cells into macrophages, lymphocytes or neutrophils also did not show any correlation with antioxidant activity in follicular aspirates. The functional role of follicular fluid inflammatory cells still remains unclear, though an immunological role in preventing development of autoimmune disease in the ovary has been suggested (Sakaguchi et al, 1985). The lack of correlation between follicular cell components and TAC or TAC loss in the present study however supports the concept

that the contribution of these cells to follicular fluid antioxidant activity is largely insignificant, indicating that follicular fluid antioxidants derive largely from plasma exudates entering via the 'blood-follicle barrier'. There are however no comparable studies to provide evidence in support of this proposal. Indeed, the proposal would be difficult confirm because it would require that antioxidant activity be compared directly between fluid in individual follicle and serum in the corresponding perifollicular vascular supply. It would be easier to correlate individual follicular fluid component with the perifollicular blood flow, determined by Doppler imaging. In one of such indirect studies, Van Blerkom et al (1997) found that levels of follicular fluid dissolved O_2 content and VEGF were positively correlated with perifollicular blood flow.

One could therefore speculate that follicular fluid cellular components do not contribute significantly to oxygen free radicals/ROS content, since an inverse relationship exists between antioxidant activity and free radical activity (Section 1.5.1). This contrasts to the situation in males, in which free radical levels in semen are positively correlated with the abundance of seminal inflammatory cells (Halliwell and Gutteridge, 1999; Whittington and Ford, 1999; Ochsendorf, 1999). The difference lies in the fact that seminal inflammatory cells are often a response to infection and tend to be activated, producing large quantities of free radicals, unlike follicular fluid which is presumably sterile. Seminal infections lead to excessive ROS production, resulting from an 'oxidative burst' from inflammatory cells as a first-line defence mechanism (Ochsendorf, 1999).

The absence of a positive correlation between the different fractions of follicular inflammatory cells and TAC or TAC loss might also indicate that these cells had no

regulatory influence on prooxidant-antioxidant balance, albeit the number of samples analysed was not enough to ensure statistically significant results. The results of the present study contrast with those of Lukassen et al (2003), who used subtype-specific fluorescent labelled monoclonal antibodies and triple colour flow cytometry to discriminate between T cell and natural killer (NK) cell subpopulations in follicular fluid, and found a shift from T to NK cells in idiopathic infertility, relative to male factor and tubal factor infertility. These authors proposed that this NK subset might negatively affect folliculogenesis and oocyte maturation, reflected by a diminished fertilisation rate in the idiopathic infertility group. The two studies were however not comparable because the inflammatory cells were not sub typed in the present study. Furthermore, it was not possible to determine the relationship between the follicular fluid cellular content and early reproductive outcome because of the small number of samples examined cytologically.

Chapter 6

Summary and Discussion

Outline

In this concluding chapter, the summaries of the studies described in this thesis are brought together. The hypothesis and aims listed in Section 1.6 of the introduction chapter are re-evaluated based on the results of the studies. Attempts are made to provide explanations for the relationships which emerged, and to relate some of the findings to the clinical situation.

Infertility affects approximately 15% of the reproductive population in the developed world and often causes emotional stress that may adversely affect the relationship of couples who wish, but fail to have, children. In some infertile couples, aetiology is unknown ('unexplained'). It is important to explain the possible mechanisms by which the more subtle aetiological and confounding factors, such as PCO and cigarette smoking, influence fertility. Specifically, this thesis tested the hypothesis that follicular fluid oxygen free radicals/ROS and antioxidants influence female reproductive performance.

The current literature regarding the role of oxygen free radicals and ROS in human female reproduction is inconsistent. Furthermore, the therapeutic role of exogenous antioxidants in human reproduction also remains controversial (Paszkowski et al, 1996; Kessopoulou, 1995), and yet antioxidant vitamins are already being given to infertile women, including antioxidant supplementation of embryo culture medium.

It would have been ideal if follicular fluid oxygen free radical concentrations could have been measured directly, however this was difficult for us since free radicals are such dynamic and unstable entities. Furthermore, the use of a specific by-product of free radical reactions would not accurately reflect the total free radical activity in the

system, since no by-product is produced in common by all free radicals and ROS. In common with many studies therefore, antioxidant capacity was assessed as a surrogate for free radical and ROS activity. Estimation of follicular fluid TAC and TAC loss over 72 h were thought to be appropriate as indirect indicators of the activity of oxygen free radicals and ROS for the purpose of this study. It was acknowledged that no single assay for antioxidants estimates all the antioxidants present in a given sample since estimated values are dependent on the reactions occurring during assays (Pellegrini et al, 2003). Measurement of TAC considered the cumulative and synergistic action of all antioxidants in follicular fluid sample (Packer et al, 1979; Stocker et al, 1986), and the additional estimation of TAC loss over 72 h provided an measure of the ability of follicular fluid to quench free radicals, which were still generated by auto-oxidation *in vitro* because of the presence of molecular oxygen and the haem group catalysing oxidative reactions. Combining two complimentary parameters (TAC and TAC loss) to assess follicular fluid antioxidant activity aimed to provide a more complete evaluation of free radical activity, in order to strengthen the conclusions derived from the results.

In Chapter 2, the TEAC and the FRAP assays for estimating follicular fluid TAC were highly correlated, though the TEAC assay produced higher TAC estimates. This was the first study to use the FRAP assay for estimating TAC in follicular fluid, and concordance was observed with previous reports, albeit in plasma, human tears and fruits, that the FRAP method of TAC assay is simple, quick to perform, cheap and reproducible (Benzie and Strain, 1999). The FRAP assay is therefore recommendable for use in future similar studies. Other comparative assay methods for TAC suffer from disadvantages including need for expensive equipment, taking much longer time to complete, or inability to analyse as many samples simultaneously.

Because the FRAP assay does not estimate the antioxidant activity of sulphydryl groups in proteins, which would have contributed to antioxidant activity, the use of this assay necessitated a further study to estimate the sulphydryl concentrations in follicular fluid samples, the values of which corresponded to about 40% of FRAP-estimated TAC values.

Chapter 3, TAC and TAC loss were determined in 303 follicular fluid samples from 34 patients undergoing IVF. The optimal follicular fluid FRAP-estimated TAC for successful IVF, at least with respect to early reproductive outcome, appears to be in the region of 0.680 mmol/l. In the preovulatory follicle, low TAC disrupts fertilisation-competence due to the reduced ability of antioxidants to buffer free radicals, causing oxidative damage to the oocyte (Van Blerkom et al, 1997). On the other hand, high TAC in the preovulatory follicle compromises subsequent embryo viability, probably due to excessive quenching of free radicals, a critical level of which may be required for oocyte maturation, and the post-ovulatory oocyte and embryo development (Blondin et al, 1997).

Low follicular fluid volume was associated with failure of fertilisation, agreeing with Nataprawira et al (1992) and Arnot et al (1995). On the other hand, Salha et al (1998) found a negative correlation between follicular volume and oocyte fertilisation-competence, although when oocytes from larger follicles were successfully fertilised, embryo quality was comparable. In the present study, follicular fluid volume had no influence on oocyte recovery and embryo viability, whereas Arnot et al (1995) reported that successful oocyte retrieval and good embryo quality were more likely with increasing follicular volume. One may therefore conclude that follicular fluid volume probably reflects the stage of follicular/oocyte maturity.

Interesting observations emerged when the influence of aetiology of infertility and other confounding factors, on follicular fluid antioxidant or oxygen free radical/ROS activity and early reproductive outcome, were investigated. For this analysis, women with male factor infertility (MF) served as 'control' because definite problems were identified in their male partners. Interestingly, compared to the other two groups, the significantly greater proportion of viable embryos observed in MF women was most probably a reflection of the optimal condition of oocytes obtained from their follicles, lending support to the consideration of this group as 'control'.

Optimal follicular fluid TAC for positive early reproductive outcome differs with aetiology of infertility. This level was set in the region of 0.680 mmol/l for the total data set (without consideration of aetiology), 0.702 mmol/l for the tubal factor and 'unexplained' infertility sets, and at approximately 0.630 mmol/l in the MF 'control' set. For instance, a value of 0.680 mmol/l would be considered too high in the MF control group, and therefore was associated with non-viable embryos. These differences probably relate to the different levels of free radicals/ROS peculiar to the different conditions. This indicates changes in follicular environment due to different aetiology, and that positive early reproductive outcome is promoted in the 'unexplained' and tubal factor sets only if a compensatory increase in TAC levels is seen, at least in the IVF setting. Despite this modification, the associations of high TAC with fertilisation-competence, and of low TAC with viable embryos are maintained. These results have important clinical implications in an IVF setting. Once fertilisation has been achieved, low TAC in follicular fluid from the source follicle may predict the viability of the embryo by the time of transfer. It would have been extremely useful to determine whether these same embryos (associated with low TAC) would have resulted in clinical pregnancy but, since more than two embryos

were transferred, it was impossible to perform this analysis. This is one of the subjects for future studies, especially in light of the move towards single embryo transfer.

Women with PCO morphology were different from the non-PCO group, exhibiting elevated, rather than low, follicular fluid TAC in association with fertilisation-incompetence and a depressed TAC with fertilisation-competence. Furthermore, unlike in the total sample set and samples from non-PCO women, embryo non-viability was associated with low TAC, rather than high TAC, in PCO women. Also, in contrast to the non-PCO group, greater TAC loss was associated with fertilisation-incompetence in PCO women. The presence of PCO morphology alone does not fulfil the criteria for the diagnosis of PCOS (Rotterdam PCOS consensus workshop, 2004). These results however show that the presence of PCO morphology alone is associated with significant differences in follicular fluid prooxidant-antioxidant balance which is different from the non-PCO group. This is not an isolated observation and is compatible with the results of numerous studies of metabolic, endocrine and haemodynamic parameters which show similar changes (albeit to lesser degree) in women with PCO even in the absence of classic symptoms of PCOS (Lakhani et al, 2002).

In the presence of PCO morphology, fertilisation-incompetent oocytes appear to be associated with greater follicular fluid antioxidant turnover and a compensatory elevated TAC. Such high TAC as was associated with fertilisation-incompetent oocyte in the PCO group may excessively quench free radicals, causing a disturbance in follicular fluid prooxidant-antioxidant balance. Embryo non-viability was however associated with low TAC, an environment of excessive free radical activity. In

clinical terms therefore, in the presence of PCO morphology, fertilised oocytes derived from follicles with high follicular fluid TAC should be preferentially transferred, albeit it remains to be confirmed that they will subsequently result in successful pregnancies. Antioxidant supplementation of the culture media following insemination of oocytes has been tried in unselected group of IVF patients with conflicting results. One may however speculate that antioxidant supplementation restricted to women with PCO morphology may improve embryo viability.

The mechanisms for the modification of prooxidant-antioxidant balance the PCO group are not clear and will therefore require further study, but may be related to any of the associated disorders including, increased androgen index, hyper-insulinism, and tendency to ovarian hyperstimulation and/or anovulation. The effects of this modification may manifest at a later stage of female reproduction, than the time of embryo transfer considered in the present study, since PCO morphology had no significant influence on the frequency of early IVF outcomes.

Although follicular fluid TAC was similar in women aged $\leq$37 years and the older group, TAC loss was generally greater in the older women compared with the younger group. This increased antioxidant consumption in the older group had no influence on reproductive outcome, though an effect may manifest at a later stage during embryonic/foetal development to explain the widely known decline in female reproductive performance with ageing (Klein and Sauer, 2001; NICE Guideline-Fertility, 2004). Future studies to compare antioxidant supplementation of culture media between women $\geq$37 years and those <37 years may confirm an improvement in reproductive outcome in the older women.

The suggestion that smoking may have adverse influence on reproductive outcome through elevated follicular fluid free radicals was not supported by the data in this thesis. Smokers had elevated follicular fluid TAC compared with non-smokers, and reproductive outcome was not adversely affected by smoking. This was an unexpected finding because many studies had reported increased free radical activity in the blood of smokers, which has been linked to impaired fertility (Borish and Pryor, 1987; Zhou et al, 2000; Paszkowski et al, 2002). Indeed an inhibitory action of nicotinic alkaloids on granulosa cell steroidogenesis was suggested as one of the mechanisms for impaired folliculogenesis in smokers (Barbieri et al, 1986). Alho and Leinonen (1999) however found no difference in plasma antioxidant activity between smokers and non-smokers. Furthermore, even if elevated markers of ROS are found in the blood of smokers, it may not directly translate to increased levels in the follicular environment. Based on the results of this thesis, one may speculate that smoking, as a compensatory response to oxidative stress, may induce a high follicular fluid TAC. Such paradoxically elevated TAC has been reported in plasma in conditions associated with oxidative stress, such as kidney failure, metabolic disorders, and after strenuous physical exercise (MacKinnon et al, 1999; Sahlin et al, 1991). As plasma uric acid increases in these conditions, increase in TAC may occur because uric acid contributes significantly to plasma TAC. The small number of smokers in the present study precludes further statistical analysis to determine the effect of aetiology or confounders on follicular fluid TAC.

Follicular fluid TAC was comparable between fertile and sub-fertile women in natural cycles. The results however indicate a higher TAC in these women compared to the IVF patients, suggesting hormonal influences in the modulation of follicular fluid prooxidant-antioxidant balance. It is also possible that the higher TAC levels

associated with natural cycles, unlike stimulated IVF cycles, was a result of sharing of antioxidants available within the ovary among many follicles in the IVF group. It is theoretically possible that this generally lower TAC level in IVF cycles could relate to the 'poor' outcome of IVF treatment, though the small number of women in the natural cycle study remains an important caveat.

Assessment of follicular fluid sulphydryl content indicated that these entities amount to about 40% of the magnitude of the FRAP estimated TAC value, and this agrees with Wayner et al (1985) that sulphydryls contribute substantially to antioxidant activity. The mean follicular fluid total protein concentration of follicular fluid is similar to those reported by several authors (Manarang-Pangan and Menge, 1971; Johnson, 1973; Shapira et al, 1996; Carbone et al, 2003). The lack of correlation between follicular fluid TAC and protein concentration meant that it was unlikely that there was significant accidental dilution of follicular fluid with flush buffer at the time of oocyte retrieval, in which case both protein and FRAP values would have been similarly decreased. Interestingly, since a good correlation exists between sulphydryls content and TAC, the influence of sulphydryls on reproductive outcome may be similar to those described for antioxidants in Chapter 3, further validating the conclusions therein.

The study described in Chapter 5 was designed to investigate the relationship between the cellular components of follicular fluid and follicular TAC or TAC loss. This study tested the suggestion that follicular antioxidant activity and oxygen free radicals are influenced by different cell types within the fluid, which may impact on early reproductive outcome. The lack of correlation between the different cells and antioxidant activity suggests that follicular antioxidants derive largely from plasma,

rather than follicular cells. Although different subsets of follicular cells have been related to oocyte quality and reproductive outcome (Lukassen et al, 2003), these associations could not confirmed in the present study. In any case, even if different cell types were correlated with reproductive outcome, this study clearly demonstrated that the relationship was unrelated to changes in the prooxidant-antioxidant balance, because of the lack of correlation between follicular fluid cellular components and antioxidants.

The results of the study described in this thesis are in accordance with the hypothesis that follicular fluid oxygen free radicals, ROS and antioxidants influence female reproductive outcome and specifically suggest that an optimal follicular fluid prooxidant-antioxidant balance exists for successful female reproductive performance, a balance that varies with the aetiology of infertility, confounding factors such as smoking, oocyte maturation, and embryonic/foetal developmental stages. Further studies will be required to clarify the physiological and pathological role of these entities at different stages of the human female reproductive cycle. The results of such studies could potentially provide options for the improvement of fertility in the human. One major difference between the results of the present study and similar studies in the literature is the recognition that successful reproductive outcome is dependent on an appropriate prooxidant-antioxidant balance which is dependent on aetiology of infertility and confounding factors. Failure to recognise this provides the main explanation for some of the conflicting results. Evidence available in the literature provides a confusing picture of the mechanisms by which oxygen free radicals and ROS influence human female reproductive outcome. Discrepancies between results of different studies are related to methodological differences, different biological samples studied, antioxidants/antioxidant enzymes

examined, or the final outcome measures as discussed in Section 1.5.3 of the introduction.

The results from the studies in this thesis must be interpreted with caution since follicular fluid TAC and TAC loss (antioxidant consumption) were assessed as surrogates for free radical activity. Furthermore, the relatively small numbers of subjects in the study involving women in their natural menstrual cycles limit the power of this study to detect a difference between the two groups. The final outcome measure in the present study was embryo viability rather than pregnancy, making it rather difficult to directly compare with some studies that assessed pregnancy as final outcome. The recent move towards replacement of single embryo would overcome this problem in future studies. It should also be emphasised that the follicular fluid samples analysed in this study were obtained from stimulated follicles, and antioxidant or free radical activity as related to follicular fluid cellular components might not be the same as in non-stimulated follicles.

The effect of oxygen free radicals/ROS in relation to female reproductive function is complex and not fully understood. Oocytes and embryos also probably have different sensitivities to oxygen free radicals at different stages of development (Morales et al, 1999). Vitamins and antioxidant supplements are however commonly prescribed to infertile couples (Kessopoulou et al, 1995; Suleiman et al, 1996; Donnelly et al, 1999). Furthermore, vitamins C and E and selenium are often used to treat male factor infertility but antioxidant levels do not appear to be reduced in these men, and the effectiveness of the treatment remains controversial (Kessopoulou et al, 1995; Tarin et al, 1994; Suleiman et al, 1996; Donnelly et al, 1999). These practises occur despite the limited understanding regarding free radicals, antioxidants and female and

male fertility, and the lack of credible evidence to support their use. Indeed, a huge business has developed around the promotion and supply of 'fertility supplements' (containing mainly antioxidants), which exploits the desperate circumstances in which infertile couples find themselves. Much of this business is based on the internet, indeed, a search on www.google.co.uk/ co-utilizing the terms; vitamin, supplement, infertility and buy, resulted in *ca.* 752,000 hits on websites updated in the last 3 months. Some websites may contain learned academic studies: these are thought to number approximately 380, *i.e.* the number of hits on PUBMED using the search terms vitamin and infertility. It is possible that each PUBMED article may cause multiple hits, but none were seen in the first 300 hits of the www.google.co.uk/ search. Suffice to say that the vast majority of the 752,000 websites discussing 'vitamin, supplement, infertility and buy' are not peer reviewed research articles. In this environment, the practise of evidence-based medicine is alien since there is limited or absent objective review of website content related to treatment efficacy. Thus bold and often misleading statements can be made, such as 'nutritional supplements have taken center stage in the field of infertility therapy and fertility health' and 'supplements that help enhance both women's and men's reproductive and fertility health', to promote sales of their products (Pregnancy-info.net; Pregnancystore.com; Foresight.org.uk).

This study suggests that a fine prooxidant-antioxidant balance is required for optimal female reproductive performance, which appears to alter depending on the stage of oocyte maturation and embryo development, and on the aetiology of infertility and other individual factors such as PCO, age and smoking habits. Inappropriate antioxidant supplementation has the potential to interfere with this prooxidant-antioxidant balance, further compromising their fertility. For instance, because

ascorbate can also exhibit pro-oxidant properties, potentially mutagenic genetic lesions may occur even at the usual supplementation dose (Podmore et al, 1998). Supplementation may therefore potentially interfere with the optimal prooxidant-antioxidant balance in an individual, in relation to their aetiology of infertility and other confounding factors, thereby adversely affecting their fertility.

It used to be assumed that vitamin supplementation could have no harmful effect, thus the industry selling and promoting vitamin supplements arose with limited, if any, regulation. This study suggests this assumption is incorrect. The optimal follicular prooxidant-antioxidant balance varies depending on the stage of oocyte maturation, and on the aetiology of infertility and other individual factors such as PCO, age and smoking habits. Antioxidant supplementation for the purpose of improving fertility in women cannot yet be justified therefore, since no attempt is made to tailor treatment to the patient, to induce the individual's optimal prooxidant-antioxidant balance. Indeed, the present supplementation methods may decrease female fertility in some cases. More research is required before such tailored supplementation can be achieved and the claims made for the effects of vitamin supplementation on female infertility are substantiated.

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